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Identification and characterization of farR and farE as a regulator and effector of fatty acid resistance in Staphylococcus aureus

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Abstract

Although *Staphylococcus aureus* is exposed to antimicrobial fatty acids on the skin, in nasal secretions and in abscesses, specific mechanisms for regulating gene expression and intrinsic resistance in response to these fatty acids have not been reported. Through *in vitro* selection for increased resistance of *S. aureus* to linoleic acid, I identified fatty acid resistant clone FAR7, where a single nucleotide polymorphism caused a $His_{121}Tyr$ substitution in an uncharacterized member of the TetR family of transcriptional regulators, which is divergently transcribed from a gene encoding a member of the resistance-nodulation-division superfamily of multi-drug efflux pumps. I named these genes *farE* and *farR*, for *r*egulator and *e*ffector of *f*atty *a*cid *r*esistance, respectively. *S. aureus*Δ*farER* exhibited loss of inducible resistance to linoleic acid, and although FarR is a TetR family regulator which typically repress expression of a divergent gene, I found that FarR is needed to induce *farE*. Compared to wild type *S. aureus*, FAR7 exhibited increased expression of *farR* and *farE* under non-inducing conditions, and a significantly higher induced level of *farE*. Electrophoretic mobility shift assays revealed a FarR binding site in the *farER* intergenic segment, that overlaps with the +1 transcription start site of *farR* as determined by 5′-RACE. The variant FarR7 produced by *S. aureus* FAR7 failed to bind to this operator site, and nucleotide substitutions within the operator abolished binding of native FarR. Conversely, FarR and FarR7 bound equally well to a second operator site upstream of the predicted *farE* promoter. Therefore, like other TetR regulators, FarR represses its own expression, and a $His_{121}Tyr$ substitution in FarR causes a loss of auto-repression and increases expression of both *farR* and *farE*. My data reports the first description of a specific mechanism of inducible resistance to antimicrobial fatty acids in a Gram-positive pathogen and defines a new paradigm for regulation of a divergently transcribed gene by a TetR family regulator.

Keywords

Co-Authorship Statement

All studies presented in this thesis were completed by Heba Alnaseri in the laboratory of Dr. Martin McGavin with assistance from co-authors as listed below. Dr. Martin McGavin contributed to the conceptualization, design, data analysis and manuscript preparation of all experiments.

Chapter 2: Inducible expression of a resistance-nodulation-division-type efflux pump in *Staphylococcus aureus* provides resistance to linoleic and arachidonic acids.

This chapter has been previously published:

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Benjamin Arsic selected the fatty acid resistant clones that were sent for comparative genome sequencing. James Schneider and Zachariah Scinocca assisted in the *farE* reporter gene assays. Julie Kaiser assisted with the set-up of [^{14}C]linoleic acid uptake assays.

Chapter 3: Identification of FarR binding site responsible for auto-repression and efflux-mediated resistance to antimicrobial fatty acids in *Staphylococcus aureus*.

This chapter is being prepared for submission to a peer-reviewed journal.

Katherine A. Ferguson assisted with *farR* reporter gene assays and western blotting. James Schneider constructed the USA300Δ*fakA* mutant and helped with *farE* reporter gene assay in this background.

Epigraph

 "You may encounter many defeats, but you must not be defeated. In fact, it may be necessary to encounter the defeats, so you can know who you are, what you can rise from, how you can still come out of it."

-Maya Angelou

Dedication

To the remarkable woman who gave up on her dreams so I can have mine; this is for you mom.

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Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

Δ	Delta for deletion
Δ*fakA*	*fakA* deletion
Δ*farER*	*farER* deletion
Δ*tet38*	*tet38* deletion
Δ*fakA*-Δ*farER*	*fakA* and *farER* double deletion
α toxin	Alpha toxin
β-defensin	Beta defensin
β hemolysin	Beta hemolysin
β -lactamase	Beta lactamase
δ toxin	Delta toxin
γ toxin	Gamma toxin
μCi	microcurie
μg	microgram
μL	microlitre
μM	micromolar
μm	micrometre
°C	Degrees Celsius
xg	times gravity
5′-RACE	5′- rapid amplification of complementary DNA ends
AAP	Abridged anchor primer
ABC	ATP-binding cassette
ACME	Arginine catabolic mobile element
Acr	Acriflavine resistance
agr	accessory gene regulator
AmtR	Regulator of ammonium uptake
ATP	Adenosine triphosphate
AUAP	Abridged universal amplification primer
bp	basepair
BSA	Bovine serum albumin
C	Carbon
CA-MRSA	Community-acquired methicillin-resistant *Staphylococcus aureus*
Cd	Cadmium
cfu	Colony forming units
Cm	Chloramphenicol
cme	Campylobacter multidrug efflux gene
DDT	Dithiothreitol
Dha	Dihydroxyacetone
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
Erm	Erythromycin
EthR	Ethionamide repressor
fab	Fatty acid biosynthetic gene

Fad	Fatty acid degradation
Fak	Fatty acid kinase
FAME	Fatty acid modifying enzyme
FapR	Fatty acid and phospholipid biosynthesis regulator
FAR	Fatty acid resistance
FarE	Effector of fatty acid resistance
FarR	Regulator of fatty acid resistance
FarR7	FarR protein from the variant FAR7 clone
FAS	Fatty acid synthesis
G3P	Glycerol-3-phosphate
Geh	Glycerol ester hydrolase
GlcNAc	N-acetylglucosamine
GSP	Gene specific primer
HA-MRSA	Hospital-associated methicillin-resistant *Staphylococcus aureus*
H	Histidine
HapR	Regulator of HA/protease gene in *Vibrio cholerae*
His	Histidine
Ica	Intercellular adhesion
IgG	Immunoglobulin G
IPTG	Isopropyl 1-thio-β-D- galactopyranoside
IR	Imperfect repeat
IRDye	Infrared dye
Isd	Iron-regulated surface determinant
KCl	Potassium chloride
Kb	kilobase
LA	Linoleic acid
Lac	Los Angeles County clone
LB	Luria Bertani
Luk	Leukocidins
LysR	Lysine regulator
M	Molar
ManNAc	N-acetylmannosamine
Mar	Multiple antibiotic resistance
MATE	Multidrug and toxic compound extrusion
MFS	Major facilitator superfamily
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
mL	millilitre
MLST	Multi-locus sequence type
mM	Millimolar
MMPL	Mycobacterium membrane protein large
$MnCl_2$	Manganese (II) chloride
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSA	Mannitol Salt Agar
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MSSA	Methicillin-susceptible *Staphylococcus aureus*

Mtr	Multiple transferable resistance
MurNAc	N-acetylmuramic acid
NaCl	Sodium chloride
ns	Non-significant
OD	Optical density
OP	Operator
OP$_{Act}$	Activation operator site
OP$_{Rep}$	Repression operator site
P	Promoter
P$_{farE}$	*farE* promoter
P$_{farR}$	*farR* promoter
PA	Palmitoleic acid
PAGE	Polyacrylamide gel electrophoresis
PAL	Pseudo-palindrome
PBP2a	penicillin-binding protein 2a
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PHYRE	Protein homology/analogy recognition engine
Pls	Phosphate acyltransferase
pmol	picomole
poly[d(I-C)]	Poly-deoxyinosinic-deoxycytidylic acid
psi	Pound-force per square inch
PtdIns	Phosphphotidylinositol
PVDF	Polyvinylidene difluoride
PVL	Panton-valentine leukocidin
Qac	Quaternary ammonium antiseptic compounds
RBS	Ribosomal binding site
RLU	Relative light units
RND	Resistance nodulation cell division
rot	Repressor of toxin
RPM	Rotation per minute
RutR	Regulator of pyrimidine utilization operon
sar	Staphylococcal accessory regulator
sae	*S. aureus* exoprotein
SCC*mec*	Staphylococcal cassette chromosome
SczA	Streptococcus zinc-sensing regulator
SdiA	Regulator that suppresses cell division inhibitors
SDS	Sodium dodecyl sulfate
SMR	Small multidrug resistance
SNP	Single nucleotide polymorphism
SoxS	Superoxide response regulon transcriptional activator
SspA	Staphylococcus serine protease
T7SS	Type VII secretion system
TBE	Tris-borate-EDTA
Tet	Tetracycline
TFR	TetR family of transcriptional regulators

TSA	Tryptic soy agar
TSB	Tryptic soy broth
TSS	Transcription start site
Tyr	Tyrosine
uFFA	Unsaturated free fatty acid
UTR	Untranslated region
V	Volt
Y	Tyrosine

Chapter 1

1 Introduction

1.1 *Staphylococcus aureus* Overview

Staphyloccous aureus is a Gram-positive bacterium from the Firmicutes phylum that is considered as a pathobiont. It colonizes ~30% of the human population asymptomatically, and thus is a part of the resident microbiota, but also a pathogen proficient in causing diverse cutaneous and systemic infections of ranging severity (1). In his attempt to examine the underlying cause of blood-poisoning in 1881, Alexander Ogston observed micrococci with a spherical outline that stained a uniformly deep violet and grouped into clusters. He examined a series of chronic and acute abscesses and found them to contain micrococci mingled with pus. When these micrococci were injected into subcutaneous tissue of test animals, they were capable of proliferating, forming inflammatory knots, invading the tissue, and disseminating into the blood stream. In 1882, these micrococci were named *Staphylococcus* (2–4). Since this first description as a causative agent of human infection, *S. aureus* has been and continues to be a leading cause of human infectious morbidity and mortality. Over the past few decades, community-acquired methicillin-resistant *S. aureus* (CA-MRSA) strains have become a significant disease burden on healthcare systems worldwide (5). These strains display hyper-virulence and effective host-to-host transmission at a relatively minor fitness cost, posing a serious threat to public health systems (6). In fact, MRSA accounts for 78% of the skin and soft tissue infections presented to emergency departments in eleven cities in the United States; 98% of these infections being caused by the community-acquired MRSA (CA-MRSA) clone USA300, which is the strain of choice in our studies (7).

1.2 Emergence of Methicillin-Resistant Community-acquired *Staphylococcus aureus*

Prior to the debut of penicillin, the mortality rate of *S. aureus* bacteremia patients was as high as 80% (8), and as early as two short years after the introduction of the antibiotics, resistant strains of *S. aureus* resistant began to emerge (9). Penicillin-resistant *S. aureus* isolates, owing to the production of β-lactamases, were reported in the healthcare and community settings in the 1950s (10). Subsequently, methicillin use was introduced into clinical practice, but the resilient *S. aureus* developed methicillin resistance rapidly (11, 12). MRSA infections represent a significant burden on the healthcare system, and are

found to be associated with increased length of hospitalization (13, 14). In a survey of staphylococcal infections across the United States, Canada, Latin America, Europe, and the Western Pacific region, MRSA was found to be the most prevalent cause of bacteremia, pneumonia, and skin and soft tissue infections (15). For decades, MRSA infections were mostly limited to healthcare settings such as hospitals and nursing homes; however, in the late 1990s community-acquired isolates were first reported. The prevalent clinical presentations were mainly fatal necrotizing pneumonia, septicemia, and pulmonary abscesses (16, 17). There were five CA-MRSA strains responsible for the majority of staphylococcal disease worldwide; the Midwest Clone, the Southwest Pacific Oceania Clone, the European Clone, the Pacific Clone, and the USA300 clone (18). USA300 was named after the unique profile of its pulse-field gel electrophoresis (19). This clone which has been responsible for severe disease outbreaks in the past (17, 20), is currently the predominant strain of *S. aureus* in North America, and accounts for 98% of infections presented to emergency departments (7, 21).

1.3 *Staphylococcus aureus* colonization

S. aureus colonizes the skin and mucosal surfaces such as the nose and throat, as well as the axillae, vagina, and perineum; however, the preferred site of colonization seems to be the anterior nares (22–25). *S. aureus* binding to mucin is an important factor in the organism's ability to colonize the nasopharyngeal mucosal surfaces (26). Approximately 30% of the population are transient carriers, and 20% are persistent carriers of *S. aureus* in the anterior nares (27). Moreover, there is a greater possibility of recovering *S. aureus* from other body sites among these carriers compared to non-carriers (1). In fact, self-infection in carriers of *S. aureus* has been reported as early as in the late 1950s. Williams *et al.* in 1959 noticed that infectious staphylococcal strains were normally the same strains that colonized the nares in the same individual (28). Additionally, Von Eiff *et al.* in 2001 reported that 82% of their sampled bacteremia patients displayed diseases caused by staphylococcal strains of endogenous origin (29). Strains obtained from the nares of these patients were identical to those in the blood up to 14 months later (28, 29).

Another target of *S. aureus* colonization, and subsequent infection, is the skin that represents the residence of multiple microorganisms including bacteria, viruses, fungi and

even mites (30–32). The microbiome that makes up the skin is more variable over time when compared to that of the gut and mouth (33). The skin microbiome can vary with topography, thickness, and frequency of cutaneous appendages such as sweat, sebaceous glands, and hair follicles (34). Sebum, for instance, is a lipid-rich antibacterial coating that lubricates and protects the skin. It is secreted from the sebaceous glands and differentiated keratinocytes in the stratum corneum of the skin. These sebaceous glands are associated with the hair follicles, and support anoxic conditions allowing for selection of facultative anaerobic microorganisms. Studies have shown that *Propionibacterium acnes*, for instance, can grow in sebaceous glands and secrete lipases that break down sebum lipids (35). Sebum triglycerides undergo enzymatic hydrolysis by *P. acnes* resulting in the release of free fatty acids on the skin (36, 37). Additionally, hormone production, life style choices such as occupation and antibiotic use, and the genetic profile of the host also contribute to the variability of the skin microbiome (38–40). Staphylococcal species are considered one of the most stable inhabitants of the skin (41). They have developed mechanisms that enable them to inhabit moist skin microenvironments, most likely by utilizing the urea component of sweat as a nitrogen source (42). Breaching cutaneous immunity on the skin provides an opportunity for pathobionts, like *S. aureus*, to establish infections. The introduction of foreign objects, such as indwelling medical devices, also provide a niche for staphylococcal colonization. Moreover, members of the resident skin microbiota might contribute to cutaneous immunity, as was reported by Iwase *et al.* in which a subset of *Staphylococcus epidermidis* strains evolved to produce an endopeptidase protein that cooperatively works with the host's β-defensin 2 to impede *S. aureus* colonization and biofilm formation (43).

1.4 *Staphylococcus aureus* pathogenesis and clinical manifestation

Although *S. aureus* is considered a normal constituent of the human microbiota, it is also a highly successful opportunistic pathogen (1). Upon breach of cutaneous immunity, *S. aureus* excels at epithelial adhesion, invasion, and subsequently immune evasion to establish a successful infection. To accomplish pathogenesis, *S. aureus* employs a collection of virulence determinants that can be divided into three categories: secreted

enzymes; adhesion and immune evasion factors; and toxins. Secreted enzymes that are associated with *S. aureus* adhesion and invasion include: glycerol ester hydrolases that are involved in degradation of triacylglycerols; phosphphatidylinositol-specific lipase (PtdIns-phospholipase C); and enolases that mediate binding to laminin (44–49). Adhesion factors include a plethora of microbial surface proteins known collectively as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (50). The acronym was first used in 1994 to describe microbial adhesins, such as fibronectin-binding proteins A and B, collagen-binding adhesin, and *S. aureus* surface protein G/S, that span the staphylococcal wall, and have exposed domains which recognize host proteins such as fibronectin, fibrinogen, laminin, collagen, and heparin associated polysaccharides with a high degree of specificity (50–59). Subsequently, *S. aureus* is able to evade host immune defenses with factors such as staphylococcal protein A which binds immunoglobulins to block phagocytosis, and toxins such as δ toxin which is involved in neutrophil lysis and the lethal α toxin (60–70). A brief summary of *S. aureus* virulence determinants and their functions is listed in Table 1.1

Table 1.1 Summary of *S. aureus* virulence determinants.

Category	Determinant	Function	Reference
Secreted enzymes	SspA (serine protease)	Cleaves fibronectin-binding protein as well as cleaves the heavy chains of all human immunoglobulin classes.	(71–74)
	PtdIns-phospholipase C	Phosphatidylinositol-specific lipase activity that releases glycan-PtdIns-anchored cell surface proteins.	(45, 48)
	Enolase	Mediates the binding to laminin.	(45, 49)
	glycerol ester hydrolase	Involved in degradation of triacylglycerols, and impede human granulocyte function.	(44, 45)
	Adhesion factors		
	Fibronectin-binding proteins A and B	Bind fibronectin, fibrinogen and elastin.	(51–54)
	S. aureus surface protein G and S	Binds to the extracellular matrix.	(58, 59)
	Collagen-binding adhesin	Binds collagen I and IV.	(55–57)
	Elastin-binding protein	Binds to elastin.	(75, 76)
	von Willebrand factor binding protein	Binds von Willebrand factor and fibrinogen. Also binds and	(47)

Adhesion and immune evasion factors		activates prothrombin.	
	Extracellular adherence protein	Binds to extracellular matrix. Also displays immunomodulatory properties.	(77–81)
	Extracellular matrix protein-binding protein	Binds to the extracellular matrix.	(82)
	Bone sialoprotein-binding protein	Binds bone sialoprotein as well as fibrinogen.	(83–85)
	Iron-regulated surface determinants A, B, C, and H	IsdA binds to fibrinogen, fibronectin and transferrin. IsdB binds hemoglobin and hemin. IsdC binds hemin. IsdH binds haptoglobin-hemoglobin complex.	(86–92)
	Immune evasion factors		
	Staphylokinase	Involved in plasminogen activation and defensin inactivation. Displays anti-opsonic and fibrinolytic properties.	(93–96)
	Protein A	Binds immunoglobulins, tumor-necrosis factor-alpha receptor 1 and von Willebrand factor.	(61–63)
	Staphylococcal complement inhibitor	Acts on C3 convertases to inhibit complement	(97)

	Chemotaxis inhibitory protein	Binds to C5a and blocks chemotaxis	(98, 99)
	Extracellular fibrinogen-binding protein	Binds fibrinogen as well as complement C3. Recruits plasmin to degrade C3 and C3b. Inactivates complement.	(100–103)
	Capsular polysaccharides	Impede phagocytosis	(104–106)
	Multiple peptide resistance factor F	Modifies membrane phosphatidylglycerol with $_L$-lysine. Mediates resistance to cationic antimicrobial peptides and defensins as well as evasion of neutrophil killing.	(107, 108)
	Antimicrobial peptide sensing system	Two component histidine kinase and response regulator system that is involved in antimicrobial peptides resistance.	(109, 110)
Toxins	α toxin	Pore-forming cytotoxin. Stimulates inflammatory cytokines.	(66–70)
	β hemolysin	Cytotoxic action.	(111, 112)
	δ toxin	Cytotoxicty and membrane-damaging properties as well as induction of tumor necrosis factor alpha	(64, 65)

		production.	
	γ toxin	Hemolytic activity.	(113, 114)
	Enterotoxins	Superantigen with immunomodulatory properties via potent T cell activation.	(115, 116)
	Toxic shock syndrome toxin 1	Superantigen with immunomodulatory properties via potent T cell activation.	(115, 117–119)
	Leukocidins A, B, D, E and M.	LukA/B have cytolytic activity towards macrophages and neutrophils. Luk D/E/M have leukocidal activity.	(120–123)
	Panton-Valentine leukocidin	Pore-forming toxin with cytolytic activity towards macrophages and neutrophils.	(124–127)

S. aureus can cause mild skin and soft tissue infections as well as life-threatening infections such as infective endocarditis, osteomyelitis, and sepsis. In the early 1990s, these infections were primarily nosocomial, and spread rapidly in healthcare settings. In one report, a single MRSA strain spread in two hospitals in British Colombia and Manitoba within 6 weeks from the strain's introduction from the Punjab (128). First reports of CA-MRSA infections were documented in the United States in 1997, and since then CA-MRSA strains have proven to be hyper-virulent especially those belonging to the USA300 lineage (129). USA300 is particularly hyper-virulent for multiple reasons: first, it carries a smaller staphylococcal cassette chromosome (SCC*mec*) than that possessed by hospital-acquired strains, and thus has a relatively lower fitness cost (130); second, it is notorious for causing destructive infections such as necrotizing pneumonia due, in part, to the production of Panton-Valentine leucocidin (PVL) that causes leukocyte destruction and tissue necrosis (16, 131); third, it has acquired the arginine catabolic mobile element (ACME) from the skin commensal *S. epidermidis* which encodes determinants of resistance and virulence that enhance pathogenesis (132).

1.5 Genetic regulation of *Staphylococcus aureus* virulence

In addition to the cell wall associated factors, enzymes, and toxins mentioned above, *S. aureus* virulence is regulated extensively at the transcriptional level. This regulation is rather complex and involves a combination of multiple sensory and regulatory systems. The principal regulatory system in *S. aureus* is the accessory gene regulator, *agr*. The *agr* locus contains four genes, *agrBDCA*, and RNAIII which is the effector molecule of this global regulatory locus. The gene *agrB* encodes a membrane-associated protease that alters the *agrD*-encoded prepropeptide. This alteration results in the production of the small peptide molecule, which is the signal for membrane associated sensor kinase encoded by *agrC*. The recognition of this signal then results in AgrC activating the response regulator that is encoded by *agrA,* and this in turn upregulates RNAIII expression. (133–135). RNAIII governs the regulation of an array of virulence determinants, both at the transcriptional and translational levels. Additionally, there are other two-component regulatory systems and global transcriptional regulators that work with, and independent of, the *agr* system to govern the pleiotropic regulation of

staphylococcal virulence. A brief summary of those regulatory systems and the effect they exert on major virulence determinants can be found in Table 1.2

Table 1.2 Major regulators of virulence determinants in *S. aureus*.

The regulatory system	Genes regulated	The type of regulation	Reference
Accessory gene regulator *(agr)*	Protein A	-	(136)
	Fibronectin-binding protein	-	(137)
	PVL	+	(138)
	SspA (V8 serine protease)	+	(139)
	Leukotoxin LukE/D	+	(138)
	α-hemolysin	+	(140)
	β-hemolysin	+	(141)
	δ-hemolysin	+	(141)
Staphylococcal accessory regulator (*sarA*)	*agr*	+	(142)
	Protein A	-	(143)
	Fibronectin-binding protein	+	(137)
	PVL	+	(138)
	SspA	-	(139)
	Leukotoxin LukE/D	+	(138)
	α-hemolysin	+	(144)

	δ-hemolysin	+	(143)
	Toxic shock syndrome toxin 1	-	(145)
S. aureus exoprotein (*sae*)	*agr*	-	(146)
	sarA	-	(146)
	Protein A	+	(147)
	Fibronectin-binding protein	+	(147)
	SspA	+	(148)
	α-hemolysin	+	(149)
	β-hemolysin	+	(149)
	Coagulase	+	(149)
	Toxic shock syndrome toxin 1	+	(145)
Repressor of toxin (*rot*)	Protein A	+	(150)
	α-hemolysin	-	(151)

1.6 Antimicrobial Resistance mechanisms of *Staphylococcus aureus*

There are two mechanisms by which *S. aureus* acquires resistance to antimicrobials: horizontal transfer of an existing resistance gene, and through intrinsic mutations. The first evidence of resistance in *S. aureus* was to penicillin, which was mediated by β-lactamase encoded by the gene *blaZ*. Upon antibiotic exposure, a transmembrane signal transducer, BlaR1, undergoes proteolytic cleavage to inactivate a transcriptional repressor BlaI, and this de-repression permits expression of *blaZ*, encoding a β-lactamase. This enzyme hydrolyzes the β-lactam ring of the antibiotics rendering them ineffective (152, 153). The horizontal transfer of the *mec* gene accounted for the emergence of methicillin resistance among *S. aureus* strains. The *mecA* encodes the membrane-bound enzyme, penicillin-binding protein 2a (PBP2a). Typical PBPs catalyze the transpeptidation reaction required for peptidoglycan cross-linkage, and PBP2a exhibits low affinity to all agents of the β-lactam class of antibiotics. The expression of *mecA* is regulated in a similar fashion to that of *blaZ*, via the combined action of a sensor-transducer protein and a transcriptional repressor (MecR1 and MecI, respectively) (154–157). Resistance to quinolones, on the other hand, is an example of a resistance mechanism mediated by intrinsic mutation. Fluoroquinolones inhibit DNA gyrase and topoisomerase IV, which together mitigate DNA supercoiling. Mutations in the conserved quinolone resistance-determining regions reduce quinolone affinity and are associated with resistance in clinical isolates (158–160). Two resistance mechanisms to the glycopeptide antibiotic, vancomycin, have been proposed. The first mechanism is chromosomally mediated, attributed to mutations that increase synthesis of peptidoglycan and cell wall precursor, thus preventing the antibiotic from binding to its target (161–164). The second mechanism is mediated by acquisition of an enterococcal operon that is responsible for changing the terminal peptide of the cell wall precursor, thus reducing affinity for vancomycin (161–164).

1.7   *Staphylococcus aureus* envelope architecture

1.7.1   Cell envelope composition

Gram-positive bacteria are surrounded by a cell wall, composed of layers of peptidoglycan with attached accessory proteins and teichoic acids, as well as a cytoplasmic membrane. Teichoic acids can be either in the form of wall teichoic acids or lipoteichoic acids. Wall teichoic acids are anionic polymers of teichoic acids that are covalently anchored to the peptidoglycan layer of the cell envelope, whereas the lipoteichoic acids are those embedded into the cytoplasmic membrane via a lipid moiety. Wall teichoic acids are comprised of repetitive units of ribitol phosphate or glycerol phosphate, and are linked to the N-acetylmuramic acid (MurNAc) of the peptidoglycan through a disaccharide of N-acetylglucosamine (GlcNAc)-1-P and N-acetylmannosamine (ManNAc) which is followed by glycerol phosphate units (165, 166). In contrast, lipoteichoic acids are comprised of repetitive units of glycerol phosphate with a D-alanyl ester or an α- GlcNAc on the second hydroxyl group of the glycerol, and are linked by a diglucosyl diacylglycerol anchor to the outer leaflet of cytoplasmic membrane (167). *S. aureus* utilizes the presence of wall polymers to defend itself from environmental stressors. Studies have shown that lipoteichoic acids surface-anchoring properties play an integral role in invading the microvascular endothelial cells of the brain. *S. aureus* mutants deficient in lipoteichoic acid membrane anchoring were found to be impaired in their capability to penetrate the blood-brain barrier thus reducing the chance of staphylococcal central nervous system disease (168). These wall polymers have also been linked to resistance to cationic antimicrobial peptides and cationic antibiotics (169, 170).

Gram-positive bacteria are also surrounded by a single cytoplasmic membrane. The barrier function of the membrane is mediated by its phospholipid bilayer that surrounds and protects the cytoplasm. There are three major polar phospholipids in *S. aureus* membranes: phosphatidylglycerol; lysyl- phosphatidylglycerol; and cardiolipin, which is the major phospholipid during stationary phase (171). Phospholipid modifications play a vital role in resistance to host defenses and antimicrobial substances. Failure to modify phosphatyidylglycerol with L-lysine, to form lysyl-phosphatidylglycerol, reduces the positive charge of the cell envelope, rendering *S. aureus* more susceptible to cationic

antimicrobial peptides (107). Furthermore, membrane phospholipids composition must be altered to accommodate environmental stressors such as fluctuations in temperature and pH, and exogenous antimicrobials.

1.7.2　　Membrane homeostasis

Fatty acids play important roles in microorganisms, both as an integral component of cellular membranes, as well as a source of energy. While the biosynthetic pathways for these fatty acids are rather complex and energetically expensive, a stable membrane composition must be maintained. The Gram-negative *Escherichia coli* utilizes the combination of transcriptional activators and repressors, belonging to the TetR and GntR families of transcriptional regulators, to control genes involved in fatty acid metabolism in order to maintain membrane homeostasis (172, 173). This homeostasis especially must be maintained upon exposure to exogenous fatty acids that could interfere with membrane fluidity. Host-derived fatty acids are either utilized as a precursor in the membrane phospholipid biosynthetic pathway or degraded via the β-oxidation pathway to be utilized as an energy source for the bacteria. Fatty acid biosynthesis is regulated via the TetR family regulator, FabR, and fatty acid degradation is regulated via the GntR family regulator, FadR (172, 174). FadR is a repressor of the β-oxidation pathway (*fad* regulon) and an activator for the *fabA* and *fabB* genes, which in turn are under the regulation of the repressor FabR, and acyl-CoA thioesters are the ligand that modulates binding of FadR and FabR to their operator DNA (173–175). Therefore, membrane lipid homeostasis in *E. coli* is a product of a transcriptional regulator with dual functionality that contributes to both fatty acid synthesis and degradation. An overview of the regulation of lipid homeostasis in *E. coli* can be seen in Figure 1.1

In the absence of acyl-CoA

Repression of *fad* genes involved in fatty acid degradation (β-oxidation) pathway

Activation of *fab* genes involved in fatty acid biosynthesis pathway

In the presence of acyl-CoA

Activation of *fad* genes involved in fatty acid degradation (β-oxidation) pathway

Repression of *fab* genes involved in fatty acid biosynthesis pathway
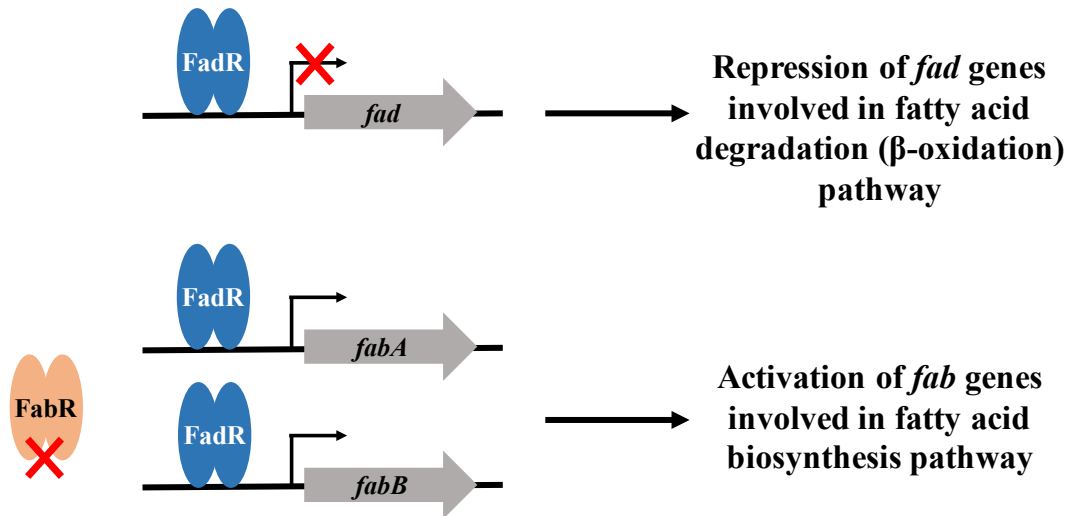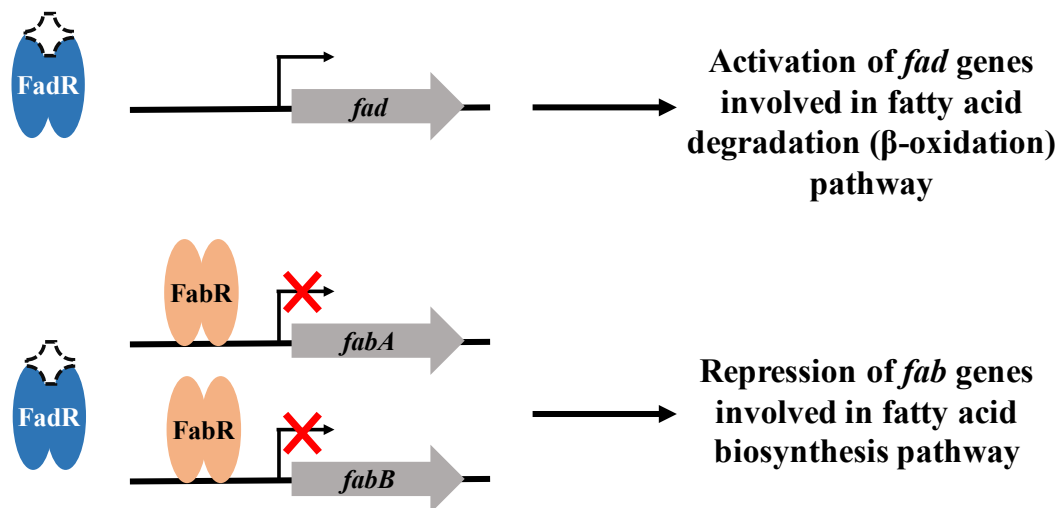
Figure 1.1 Regulation of lipid homeostasis in *E. coli*. Exogenous fatty acids are converted to acyl-CoAs after entry into the cell. In the absence of acyl-CoA, the transcriptional regulator FadR acts as a repressor of the *fad* regulon to repress fatty acid degradation, and as an activator of *fabA* and *fabB* to activate fatty acids biosynthesis. The genes *fabA* and *fabB* are also under the control of the transcriptional repressor FabR. In the presence of acyl-CoA, FadR dissociates from its operator DNA, resulting in the expression of *fad* genes to activate fatty acid degradation, as well as repression of the *fab* genes to repress fatty acid synthesis (176, 177).

Moreover, optimal membrane fluidity is governed by the fatty acid composition of phospholipids; both saturated and unsaturated. Saturated fatty acids are produced to increase membrane rigidity, whereas unsaturated fatty acids are produced to increase membrane fluidity, as is the case when the bacteria are exposed to higher and lower growth temperatures, respectively (178). For instance, *Pseudomonas aeruginosa* employs two desaturase systems, in addition to the Fab pathway, to regulate fatty acid biosynthesis to maintain membrane homeostasis. The first system is DesA which modifies existing membrane phospholipids to accommodate fluctuations in fluidity. The second desaturase system is DesB which alters saturated fatty acids of exogenous source, such as those lipids in the pulmonary surfactant encountered by *P. aeruginosa,* to produce unsaturated fatty acids to maintain optimal membrane fluidity. DesB is regulated at the transcriptional level by DesT which is a regulator that senses environmental fatty acids, and differentiates between saturated and unsaturated acyl-CoA substrates. In the presence of unsaturated acyl-CoA, DesT binds to an operator DNA located in the promoter region of *desCB*, to repress transcription of these genes which are necessary for production of unsaturated fatty acids. Conversely, the presence of saturated acyl-CoA causes the release of DesT from the operator DNA and the subsequent expression of *desB*, which in turn introduces double bonds into the acyl-CoA products of exogenous fatty acids and restores optimal membrane fluidity (179–181).

Comparatively, transcriptional regulation of membrane lipid homeostasis in Gram-positive bacteria is less well understood. The most detailed studies have been conducted in *Bacillus subtilis*, where a two-component desaturase system encoded by *desK and desR* regulates the membrane-bound acyl desaturase, DesA. This desaturase system senses temperature fluctuations and since *B. subtilis* is incapable of *de novo* synthesis of unsaturated fatty acids, the bacterium employs DesA to desaturate existing phospholipids in order to maintain membrane fluidity (182, 183). Additionally, *B. subtilis* also employs a transcriptional regulator, FadR, which controls genes involved in fatty acid degradation. Unlike FadR of *E. coli,* which is a GntR regulator, FadR of *B. subtilis* is a TetR family regulator. Similar to *E. coli, B. subtilis* FadR is a repressor of genes involved in fatty acid degradation, and acyl-CoA is the ligand that modulates binding of FadR to its operator

DNA. In the presence of exogenous fatty acids, the promoters repressed by FadR are de-repressed and fatty acids are degraded to be utilized as a carbon source (184)

Furthermore, *B. subtilis* employs transcriptional regulation to sense the status of the intracellular pool of fatty acids to maintain lipid homeostasis and optimal membrane fluidity. The repressor FapR regulates the expression of genes involved in fatty acid and phospholipid synthesis in response to the cellular levels of malonyl-CoA. Elevated levels of malonyl-CoA are indicative of diminished fatty acid and phospholipid synthesis which in turn results in the de-repression of FapR-mediated genes that are involved in lipid biosynthetic machinery (185, 186). FapR is the first global transcriptional regulator that is highly conserved among Gram-positive bacteria (185, 187). FapR was first characterized and purified from *B. subtilis*, and was subsequently found to be conserved in other organisms such as *S. aureus* and *Listeria monocytogenes*. Similar to FapR in *B. subtilis*, staphylococcal FapR senses malonyl-CoA to monitor lipid synthesis in a feed-forward mechanism to maintain membrane homeostasis (185, 188). This feed-forward mechanism involves recognition of an upstream biosynthetic product to regulate the transcription of the genes involved in the lipid biosynthetic machinery.

A.

In the absence of acyl-CoA

FadR

fad

Repression of genes involved in fatty acid degradation

In the presence of acyl-CoA

FadR

fad

De-repression of genes involved in fatty acid degradation

B.

Low cellular pool of malonyl-CoA

FapR

fap

Repression of *fap* genes involved in fatty acid and phospholipid biosynthesis pathway
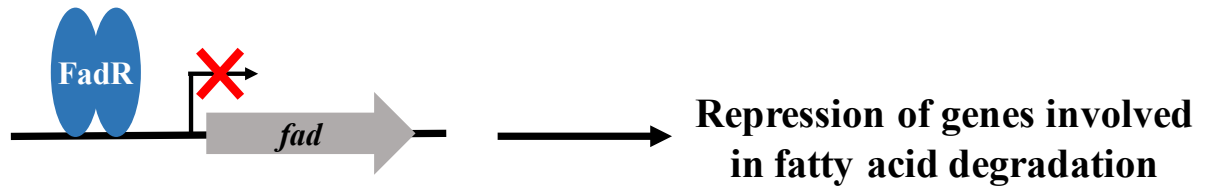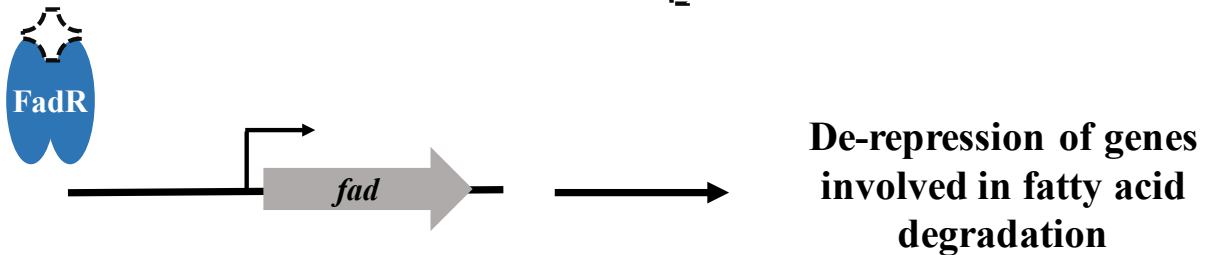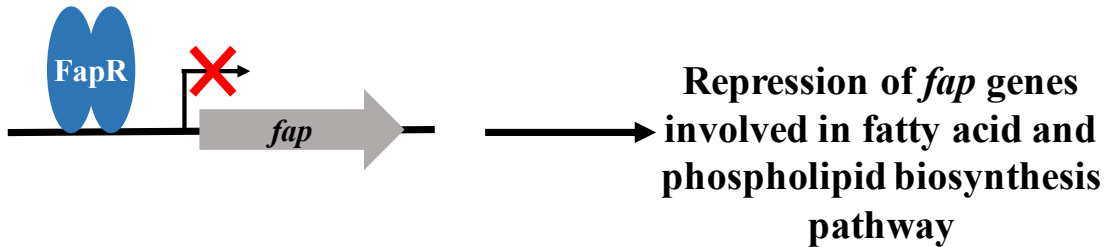
Elevated cellular pool of malonyl-CoA

FapR

fap

Relief of FapR mediated repression of genes involved in fatty acid and phospholipid biosynthesis pathway
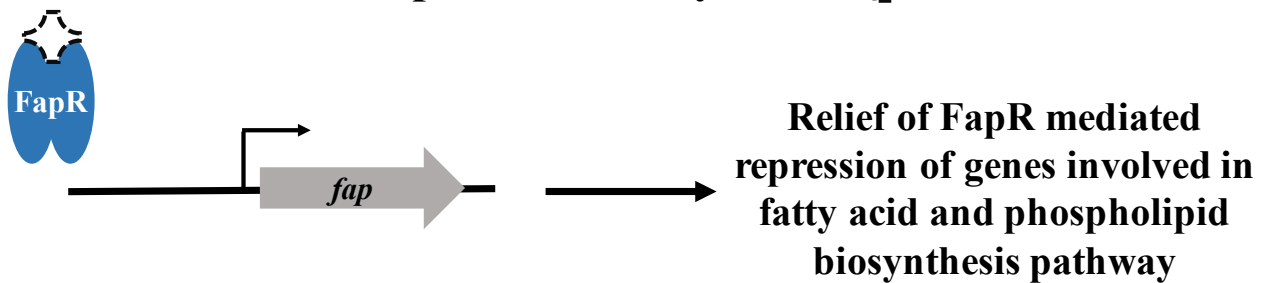
Figure 1.2 Regulation of lipid homeostasis in *B. subtilis*. A. Exogenous fatty acids are converted to acyl-CoAs after entry into the cell. In the absence of acyl-CoA, the transcriptional regulator FadR acts as a repressor of the *fad* regulon to repress fatty acid degradation. In the presence of acyl-CoA, FadR dissociates from its operator DNA, resulting in the activation of fatty acid degradation pathway to utilize the exogenous fatty acids as a carbon source. **B.** FapR is responsive to the cellular status of fatty acid biosynthesis. Malonyl-CoA is the ligand modulating FapR binding to its operator DNA. In low levels of malonyl-CoA, fatty acid biosynthesis genes are repressed. In the presence of malonyl-CoA, FapR dissociates from its operator DNA, resulting in the expression of *fap* genes to activate fatty acid and phospholipid synthesis.

1.8 Fatty acid machinery of *Staphylococcus aureus*

1.8.1 Phospholipid composition

There are three major phospholipids in *S. aureus* membranes: phosphatidylglycerol; lysyl- phosphatidylglycerol; and cardiolipin (171). These phospholipids play a crucial role in preserving membrane biophysical properties, and particularly, the fatty acid composition of phospholipids is the key determinant of membrane fluidity (189) . Unlike *B. subtilis* that possesses a gene encoding a membrane phospholipid desaturase for the synthesis of unsaturated fatty acids, *S. aureus* does not possess such desaturase and thus is incapable of producing these fatty acids (182). Instead, a combination of branched- and straight-chain fatty acids are present in staphylococcal membranes. Branched-chain fatty acids are the predominant fatty acids in *S. aureus* phospholipids, comprising 55-65% of the total fatty acids (189, 190). Branched-chain fatty acids are synthesized from branched-chain amino acids; these include leucine- and valine-derived (iso) fatty acids, and isoleucine-derived (anteiso) fatty acids, with the latter in particular promoting increased membrane fluidity (189, 191).

1.8.2 Phospholipid synthesis

The biosynthetic pathway of staphylococcal branched-chain fatty acids starts when the amino acids leucine, isoleucine and valine undergo a transamination reaction mediated by the branched-chain amino acid transaminase BAT. The resulting branched-chain α-keto acid then undergoes a decarboxylation reaction mediated by α-keto acid dehydrogenase (BKD), resulting in the formation of branched-chain acyl coenzyme A derivatives which are the precursor for fatty acids biosynthesis. The branched-chain acyl coenzyme A derivatives, in combination with malonyl-ACP, are then utilized by the β-ketoacyl-ACP synthase III (FabH) to generate β-ketoacyl-ACP. This in turn undergoes a reduction reaction mediated by FabG reductase, a dehydration reaction mediated by FabZ and a reduction reaction mediated by FabI, resulting in the formation of saturated acyl-ACP. The enzyme FabF catalyzes the condensation reaction permitting further product elongation. The resulting long chain acyl-ACP is then used in the generation of the phospholipid precursor, phosphatidic acid (189, 192–196).

The peripheral membrane protein PlsX is responsible for transferring the acyl group from Acyl-ACP to an inorganic phosphate, forming an acylphosphate product, which can then be used by an integral plasma membrane protein, PlsY. This interaction between PlsY and the acylphosphate product, in the presence of glycerol-3-phosphate, results in the formation of Acyl-G3P, which is then acylated by the integral membrane protein PlsC, forming phosphatidic acid that represents the key intermediate in membrane phospholipid formation. Phosphatidic acid is subsequently used, in the presence of cytidine triphosphate, to synthesize cytidine diphosphate diacylglycerol through the action of phosphatidate cytidylyltransferase. Phosphatidylglycerol-phosphate is then generated upon the replacement of cytidine monophosphate with glycerol phosphate. The dephosphorylation of phosphatidylglycerol-phosphate results in the formation of phosphatidylglycerol which is the core phospholipid in *S. aureus* (196–201). An overview of fatty acid and phospholipid biosynthesis in *S. aureus* can be seen in Figure 1.3

In addition to the fatty acids produced by the fatty acid synthase machinery mentioned above, *S. aureus* is also capable of utilizing fatty acids of exogenous source. Upon entry into the cell by flipping across the membrane leaflet, exogenous fatty acids are processed by the fatty acid kinase machinery. This machinery includes a kinase domain protein, FakA, and a fatty acid binding protein FakB1 and FakB2 for binding saturated and unsaturated fatty acids, respectively. Exogenous fatty acids bind FakB, and after phosphorylation by FakA, are incorporated into the phospholipid directly, or indirectly after passing through an extension cycle by the FASII machinery (202). During the extension cycle, acyl chains are extended by two carbons via four enzymatic reactions. Extension is initiated by an elongation condensing enzyme, FabF, and the resulting β-ketoacyl-ACP is then reduced by the reductase, FabG. Subsequently, β-hydroxyacyl-ACP undergoes a dehydration reaction through the action of FabZ. The resulting trans-2-enoyl-ACP undergoes a reduction reaction that is catalyzed by the reductase FabI to complete the extension cycle (203–206). The peripheral membrane protein PlsX is responsible for transferring the acyl group from the extended fatty acid product to an inorganic phosphate, forming an acylphosphate product, which can then be used by an

integral plasma membrane protein, PlsY. This interaction between PlsY and the acylphosphate product results in the acylation of glycerol-3-phosphate forming the first intermediate in membrane phospholipid formation, known as lysophosphatidic acid. Lysophosphatidic acid is then acylated by the integral membrane protein PlsC, forming phosphatidic acid that represents the core intermediate in membrane phospholipid formation (197–200). An overview of exogenous fatty acid utilization in *S. aureus* can be seen in Figure 1.4

Leucine
Valine
Isoleucine

Branched-chain amino acid transaminase

Branched-chain β-keto acids

α-keto acid dehydrogenase

Branched-chain acyl coenzyme A derivatives

+ malonyl-ACP

FabH

β-ketoacyl-ACP

FabG
FabZ
FabI

Saturated acyl-ACP

FabF

Long chain acyl-ACP

PlsX

Acyl- PO_4

+ Glycerol 3-phosphate

PlsY

Acyl- G3P

PlsC

Phosphatidic acid

Cytidine diphosphate diacylglycerol

Phosphatidylglycerol phosphate

Phosphatidylglycerol

Figure 1.3 Overview of fatty acid and phospholipid biosynthesis in *S. aureus*. Synthesis of staphylococcal branched-chain fatty acids starts when the amino acids leucine, isoleucine and valine undergo a transamination followed by a carboxylation reactions reaction, resulting in the formation of branched-chain acyl coenzyme A derivatives which are the precursor for fatty acids biosynthesis. The branched-chain acyl coenzyme A derivatives, in combination with malonyl-ACP, are then utilized by FabH to generate β-ketoacyl-ACP. This in turn undergoes a reduction reaction by FabG, a dehydration reaction by FabZ and a reduction reaction by FabI, resulting in the formation of saturated acyl-ACP. The enzyme FabF catalyzes the condensation reaction permitting further product elongation where the resulting acyl-ACP can undergo additional rounds of elongation by FabG, FabZ and FabI. For simplicity, the steps shown here are unidirectional. Subsequently, the resulting long chain acyl-ACP is then used in the generation of the phospholipid precursor, phosphatidic acid. The protein PlsX is responsible for transferring the acyl group from Acyl-ACP to an inorganic phosphate, forming an acylphosphate product, which can then be used by PlsY to form acyl-G3P. The latter in turn is acylated by PlsC, forming phosphatidic acid which is subsequently used, in the presence of cytidine triphosphate, to synthesize cytidine diphosphate diacylglycerol through the action of phosphatidate cytidylyltransferase. Phosphatidylglycerol-phosphate is then generated upon the replacement of cytidine monophosphate with glycerol phosphate, and subsequently dephosphorylated to form phosphatidylglycerol which is the core phospholipid in *S. aureus* membranes. Figure is adapted from Schiebel *et al.* and Kuhn *et al.* (193, 196).
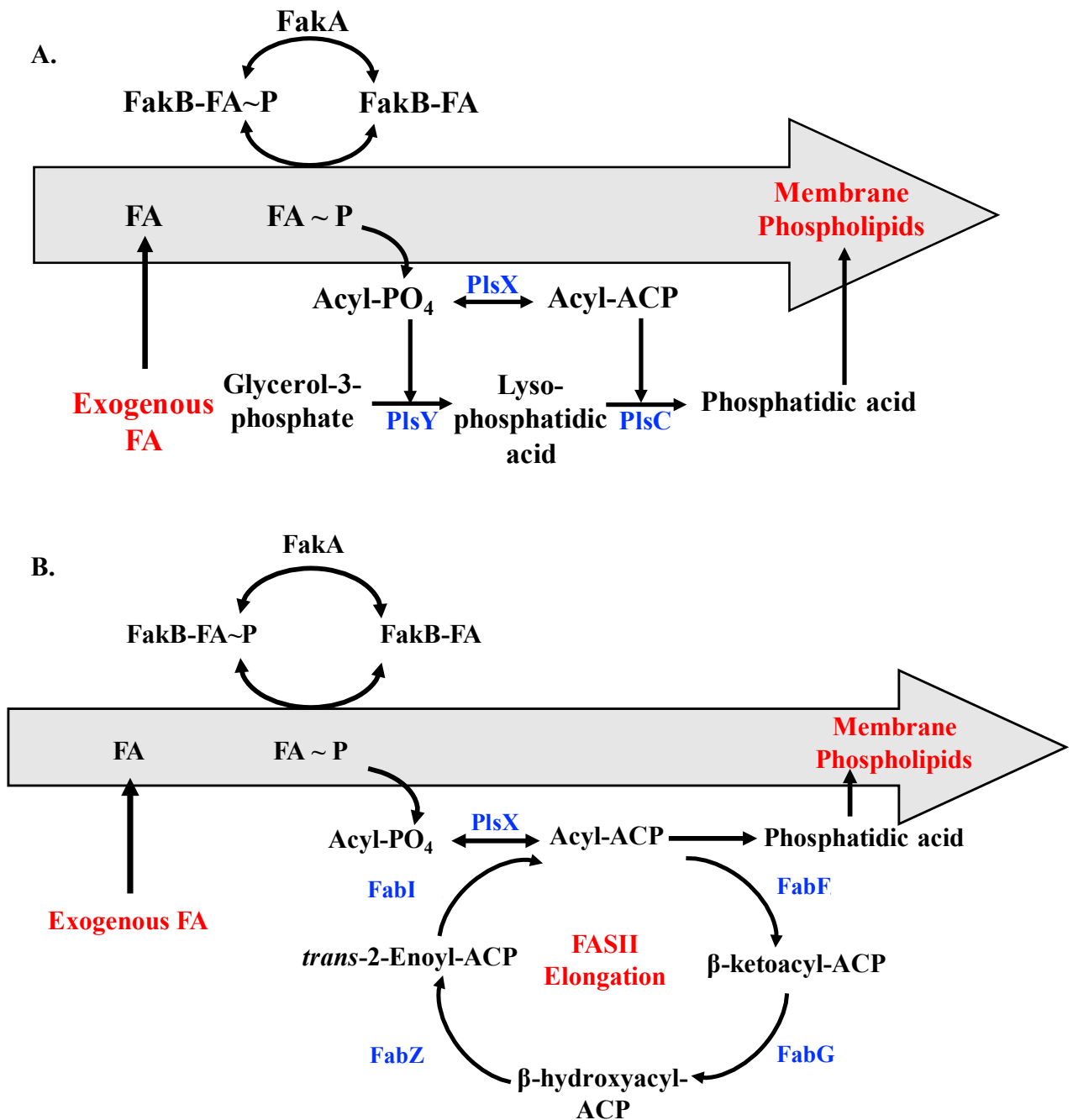
Figure 1.4 Overview of exogenous fatty acid utilization by *S. aureus*. Upon entry into the cell, exogenous fatty acids (FA) are processed by the fatty acid kinase machinery; FA are bound by FakB and phosphorylated by FakA. Subsequently, phosphorylated fatty acids are either are incorporated into the phospholipid directly (A), or indirectly after passing through an extension cycle by the FASII machinery (B). Figure is adapted from Parsons *et al.,* and Yao and Rock (202, 207).

1.8.3   Lipids as antimicrobials

The skin is the first line of defense against microbial infection. One of the host innate defense elements on the skin is antimicrobial long-chain unsaturated fatty acids. The main source of these fatty acids is the sebum secreted by the sebaceous glands and differentiating keratinocytes present in the stratum corneum of the epidermis (208). The sebum consists of squalene, wax monoesters, triglycerides, and small amounts of cholesterol and cholesterol esters (209). As the sebum's constituents stream outwards through the hair follicle associated with the sebaceous glands, the triglycerides undergo enzymatic hydrolysis to produce unsaturated free fatty acids (uFFA) on the skin surface. The principal uFFA derived from sebaceous triglycerides is sapienic acid, which is a 16-carbon fatty acid with one degree of unsaturation that has potent bactericidal property against MRSA (208). Linoleic acid was identified as the major uFFA in human nasal secretion and is also present in *S. aureus* abscesses, a hallmark of *S. aureus* skin and soft tissue infections (210–212). Humans deficient in the production of uFFAs are more susceptible to *S. aureus* skin infections (213).

Although *S. aureus* possess the genes coding for enzymes involved in fatty acid degradation, studies have ruled out their involvement in exogenous fatty acid utilization (214). Alternatively, the only metabolic fate of exogenous uFFAs is through incorporation into the membrane phospholipid (194, 215). This incorporation is the rate limiting step such that when the levels of exogenous uFFAs exceeds that of the phospholipid biosynthetic machinery, uFFAs accumulate in the membrane and become detrimental (216). Although it has been long recognized that uFFAs have membrane damaging properties (217, 218), the exact mechanism by which these uFFAs exert their toxicity remained debatable. Several mechanisms have been proposed; these include uFFAs increasing the permeability of bacterial membranes due to their surfactant action, and disrupting the anisotropic nature of membranes by interfering with protons movement across the membrane and subsequently ATP synthesis (219, 220). Other reports have proposed that the toxic effects of uFFAs are mediated through a peroxidative process and through inhibiting fatty acid synthesis by acting as selective inhibitors of the enoyl –acyl carrier protein reductase (FabI) (221, 222). However, recent reports confirm

that exogenous uFFAs disrupt the cytoplasmic membrane integrity, resulting in dissolution of the proton gradient across the membrane. This in turn leads to leakage of cellular metabolites and low-molecular weight proteins, and the eventual cessation of macromolecular biosynthetic pathways and loss of cellular viability (216).

Table 1.3 Summary of proposed mechanisms of uFFA toxicity.

Target	Mechanism	Reference
Cytoplasmic membrane	Uncouple the electron transport chain	(223, 224)
Cytoplasmic membrane	Impede oxidative phosphorylation	(220)
Cytoplasmic membrane	Surfactant action that increases membrane permeability	(219)
Cytoplasmic membrane	Increase membrane fluidity leading to cell lysis	(218, 225)
Cytoplasmic membrane	Dissolution of the proton gradient across the membrane and subsequent release of low molecular-weight proteins	(216)
Fatty acid synthetic machinery	Enzymatic inhibition	(222)
Cellular viability	Oxidative stress	(221, 226)

Although exogenous uFFAs display antimicrobial properties, bacterial species are equipped with intrinsic mechanisms of resistance to these uFFAs. External structures such as the outer membrane and cell wall of Gram-negative and positive bacteria, respectively, mediate protection against uFFAs through several mechanisms that are briefly reviewed here. First, transcriptomics and proteomics studies show that upon exposure to exogenous uFFAs, *S. aureus* upregulates genes involved in carotenoid biosynthesis, as well as those in cellular energy metabolism, peptidoglycan, and call wall biosynthetic pathways (227). Cell wall components play an integral role in *S. aureus* susceptibility to antimicrobial uFFAs, as wall structure, thickness and properties can impede the entry of these uFFAs. It has been proposed that the iron-regulated surface determinant, IsdA, is involved in resistance to uFFAs where this cell wall anchored protein modifies cellular hydrophobicity. The C domain of IsdA extends into the staphylococcal cell wall, altering its charge and hydrophobicity thus rendering the cells less susceptible to host antimicrobial peptides and fatty acids that rely on hydrophobic interactions to penetrate the cellular membrane and manifest their effects (227, 228). Other reports suggest that *S. aureus* counteracts the uFFA-mediated increase in membrane fluidity by upregulating the production of staphyloxanthin, leading to a direct correlation between membrane stability, cellular pigmentation, and uFFAs-induced cellular killing (218, 227, 229). The production of staphyloxanthin is regulated by the sigma B (σ^B) regulon as a general stress response to the presence of uFFAs (227). Additionally, it has been proposed that wall teichoic acids confer resistance to uFFAs, by acting as a filter to restrict their passage across the cell wall, in a structure-specific manner, and cells lacking teichoic acids exhibit enhanced leakage of intracellular content (216, 230). Moreover, the fatty acid modifying enzyme (FAME), responsible for fatty acid esterification, has also been linked to uFFA tolerance. FAME can esterify bactericidal fatty acids that are present in staphylococcal abscesses to various alcohols, thus enabling bacterial survival within tissues (227, 231). As is the case with the majority of antimicrobial resistance, it can be anticipated that resistance to uFFAs should also be mediated through efflux pumps. *Neisseria gonorrhoea*, for instance, exhibits an efflux system dedicated to extrusion of hydrophobic antimicrobials such as free fatty acids and bile salts (232, 233). Similarly, there are some reports of efflux pumps contributing to *S.*

aureus resistance to skin antimicrobials such as fatty acids and polyamines. Tet38 is a multidrug efflux transporter that confers resistance to tetracycline as well as palmitoleic acid, one of the main uFFA in human nasal secretion (221, 234). Tet38 is implicated in *S. aureus* survival and colonization on the skin due to its ability to transport antimicrobial free fatty acids thus conferring resistance to them (234). However, this efflux pump is not specific just for uFFAs, as it contributes to tetracycline resistance as well, and therefore a specific fatty acid efflux pump is yet to be identified in *S. aureus*.

Table 1.4 Summary of uFFA resistance mechanisms proposed to date.

Target	Mechanism	Reference
Cell wall physiology	Decrease cellular hydrophobicity and thus prevent uFFA penetration	(228)
Membrane stability	Increase staphyloxanthin production to counteract the uFFA-mediated increase in membrane fluidity	(218, 227, 229)
Wall teichoic acids	Prevent binding of uFFAs	(216, 230)
uFFA transport	Tet38-mediated efflux of uFFAs outside the cell	(234)
uFFA processing	FAME- medicated esterification of uFFAs to various alcohols	(227, 231)

1.9 Bacterial efflux pumps

One of the greatest innovations in modern medicine is the introduction of antibiotics in clinical practices to treat the otherwise life-threatening infectious diseases. However, bacterial pathogens were able to circumvent this progress by developing resistance mechanisms leading to infectious diseases that are once again, a major problem in the hospital and community settings. The World Health Organization continuously urges for the development of new bacterial therapeutic targets to combat the life-threatening multidrug resistance (235, 236).

The efficacy of antimicrobial compounds can be abrogated by a number of mechanisms including: reduced uptake; enzymatic inactivation; target site modification; and efflux from the cell (237, 238). There are five primary families of multidrug efflux pumps: resistance nodulation cell division (RND); major facilitator superfamily (MFS); ATP binding cassette (ABC) family; multidrug and toxic compound extrusion (MATE) family; and small multidrug resistance (SMR) family. In addition to their notable functions in multidrug resistance, the RND efflux pumps have been extensively studied for their roles in fatty acid efflux (239, 240). The most well characterized member of the RND family of multidrug efflux pumps is the AcrB pump of *E. coli.* AcrB uses proton motive force to extrude hydrophobic substances such as antibiotics, bile salts, and a range of cellular metabolites. This tripartite efflux system in *E. coli* is composed of the trimeric AcrB that spans the inner membrane, TolC which is an outer membrane channel, and the periplasmic adaptor protein AcrA (241–243). Another RND family membrane protein that has a role in lipid efflux is the mycobacterium membrane protein large (MMPL) group of proteins in *Mycobacterium tuberculosis*. MMPLs are co-localized with lipid-metabolizing enzymes, and are involved in the transport of methyl-branched lipids (244). MMPL-mediated lipid pathways have been implicated in bacterial intracellular persistence, and four of the MMPL proteins appear to be indispensable for *M. tuberculosis* in fully maintaining virulence in mice (244).

1.10 TetR family of transcriptional regulators and their biological functions

Transcriptional regulators are the key to most microbial adaptation strategies. These regulators normally contain a DNA- and ligand- binding domain that can be used to sense environmental signals and elicit a downstream response at the transcriptional level. There are many families of transcriptional regulators that have been grouped based on structural and functional similarities, such as the helix-turn-helix motif, which is the most common DNA-binding motif among prokaryotes (245–247). Members of these families have either an activation or a repression function, with a few families that display unconventional dual roles in activation and repression. Members of the LysR, IcIR, MarR, and Crp families of transcriptional regulators have displayed dual roles (248–251). Recently, there have been few reports of members of the TetR family of transcriptional regulators that display dual functionality (252, 253).

TetR family regulators (TFRs) have been well documented for their roles in environmental adaptation and antibiotic resistance (254). There are more than 2500 TFRs with functions ranging from multidrug resistance, biosynthesis of antibiotics, and pathogenicity in both Gram-positive and Gram-negative bacteria (255). TFRs are named after the well-characterized TetR, a repressor that controls expression of a membrane-associated protein, TetA, that exports tetracycline out of the bacterial cell. *tetR* and *tetA* are divergently transcribed, and the *tetR* gene product controls expression of both *tetA* and *tetR*. When tetracycline is present and associated with Mg^{2+}, it binds to the TetR protein leading to a conformational change that renders TetR unable to bind to the DNA. As a result, *tetR* and *tetA*, both of which are repressed by TetR, are expressed (256–260).

A well-documented staphylococcal TFR is QacR, which is a repressor regulating the expression of QacA, a multidrug efflux pump. Lyon *et al.* in 1984 showed that the *S. aureus* plasmid pSK1 encodes resistance to quaternary ammonium antiseptic compounds. This resistance is mediated by energy-dependent efflux encoded by the *qacA* gene that belongs to a regulatory circuit similar to that of the efflux-mediated resistance to tetracycline. This plasmid-encoded system also contained a transcriptional regulator with sequence similarity to the helix-turn-helix motif of the transcriptional repressor that

governs tetracycline-resistance in Gram-negative bacteria (261–264). By 1998, it was confirmed that QacA contains 14 transmembrane domains and that it belongs to the major facilitator superfamily of transporters, and that QacR is the repressor regulating the expression of this transporter (265, 266). QacR functions as a homodimer with an N-terminal DNA-binding domain recognizing an operator sequence located downstream from the divergent *qacA* promoter (266–268). This binding is considered unorthodox for a repressor as it may not physically block the binding RNA polymerase directly, but rather it hinders the transition of the transcription machinery. The regulator of intercellular adhesion (*ica*) operon of *S. epidermidis* is a TFR that is responsible for regulating the synthesis of polysaccharide intercellular adhesin (PIA) and, subsequently, biofilm formation (269). Two IcaR homodimers bind cooperatively to an operator site located upstream of the start codon of *icaA* to repress its transcription (270, 271). Upon ligand binding of streptomycin and gentamicin, IcaR dimers undergo a conformational change in the DNA binding domain, and the subsequent transcription of the divergent *icaADBC* operon (272). Cramton *et al* in 1999 showed that the *ica* locus is not only present in *S. aureus*, but is also implicated for biofilm formation (273); however, in *S. aureus*, the teicoplanin-associated locus regulator (TcaR), a member of the MarR family of transcriptional regulators, is also a repressor of the *ica* operon (272).

Other examples of TFRs include: the AmeR of *Agrobacterium tumefaciens* which is a regulator of an RND- efflux system; AmrR of *Pseudomonas aeruginosa* which is a regulator of an efflux pump involved in aminoglycoside resistance; and BpeR of *Burkholderia pseudomallei* which is a repressor of an RND pump involved in gentamycin, streptomycin, and erythromycin resistance (274–276). Similarly, resistance to the anti-tuberculosis drug ethionamide in *M. tuberculosis* is mediated by the TetR regulator EthR, and resistance to hydrophobic antimicrobial agents that target *N. gonorrhoea* is mediated by MtrR (233, 277–279). TFRs are also involved in regulation of virulence genes, such as HapR, which is a density-dependent regulator of virulence in *Vibrio cholera* (280, 281).

Additionally, several TFRs have a role in regulation of fatty acid efflux. AcrR is a repressor of the multidrug efflux pump AcrAB in *E. coli*; a prototypic member of the

RND family of efflux pumps. AcrR interacts with the *acr* promoter located in the intergenic segment between *acrR* and *acrAB*. This interaction between AcrR and the promoter region is responsive to accumulation of cellular metabolites and bile salts as well as global stress signals. In the presence of inducing ligand, AcrR is de-repressed and functions in combination with global transcriptional activators to enable efflux pump expression (282–285). Other TFRs that have roles in lipid efflux include: CmeR from *Campylobacter jejuni* and MtrR from *N. gonorrhea.* CmeR is a repressor of an RND-efflux pump, CmeABC, that is responsive to accumulation of amphipathic bile salts (286–289). Similarly, MtrR is a repressor of an RND pump, MtrCDE, the efflux of which enables gonococcal growth on mucosal surfaces that are enriched in fatty acids and bile salts. Additionally, MtrR is a repressor of a MarR family transcriptional regulator, FarR, which in turn is a repressor of the MFS efflux pump FarAB. Therefore, MtrR also functions as a positive regulator of the FarAB efflux pump which mediates resistance to antimicrobial fatty acids (233, 279, 290–292).

1.11 Rationale and hypothesis

CA-MRSA strains are particularly efficient in colonization, transmission, and causing invasive skin and soft tissue infections (6). Our laboratory strain of choice is USA300, the dominant CA-MRSA strain in the United States, accounting for 98% of MRSA infections presenting to emergency departments (293). USA300, which was isolated in September 2000, has been linked to infection outbreaks in at least 21 American states, Canada, and Europe (132). Further, this isolate is more resistant to killing by polymorphonuclear leukocytes (132). To persist on human skin, *S. aureus* must cope with uFFAs; a component of human innate defense mechanisms. Although genome sequencing and molecular typing have provided a better understanding of the pathogenic success of USA300, detailed knowledge of the specific molecular determinants, especially in regards to uFFA resistance, is still lacking (294, 295). Although *S. aureus* possess the genes coding for enzymes involved in fatty acid degradation, studies have ruled out their involvement in exogenous fatty acid utilization (214). Therefore, the only metabolic fate for exogenous uFFAs is through their incorporation into the membrane phospholipid. This incorporation step is the rate-limiting step, such that when the rate of

fatty acid import exceeds that of incorporation, excess free fatty acids accumulate in the membrane, ultimately resulting in cell death (202, 214, 216).

To better understand the factors that contribute to *S. aureus* adaptation to uFFA, our laboratory evaluated the growth of *S. aureus* USA300 in the presence of physiological concentrations of uFFA that would normally be encountered on the skin and in the anterior nares. Arsic *et al.* showed that uFFA can induce the staphylococcal proteolytic cascade (296). Additionally, Arsic *et al.* showed that unsaturated linoleic and sapienic acids are sub-inhibitory at 25 μM and bactericidal at 100 μM, whereas a 50 μM concentration caused a 10-12 hour lag phase after which bacteria resumed growth (296). It was established that the profile of secreted proteins and virulence factors is influenced by exogenous uFFAs, suggesting a possible regulatory mechanism of virulence in response to uFFAs.

To identify genes that may confer intrinsic resistance to uFFA, *S. aureus* USA300 was selected for growth at elevated concentrations of linoleic acid after which single colonies were selected for DNA isolation and genome sequencing on the Ion Torrent Platform to identify single nucleotide polymorphisms (SNP) in 7 fatty acid resistant (FAR) clones. Two of these clones contained SNP causing a His_{121}>Tyr substitution in *SAUSA300_2490* gene. Using domain enhanced lookup time accelerated BLAST, the amino acid sequence of *SAUSA300_2490* had 99% homology to TFRs and structure prediction using homology modeling server, Phyre2, predicted that *SAUSA300_2490* shares 99.8% amino acid sequence similarity with TFRs such as FadR, a regulator of fatty acid degradation in *Thermus thermophilus*.

TFRs typically exert their effect on divergent genes. Bioinformatics analyses revealed that *SAUSA300_2489*, which is divergently transcribed from *SAUSA300_2490*, encodes a gene product that belongs to the Resistance-Nodulation-Cell Division (RND) superfamily of proteins, which promote proton-antiport dependent efflux mechanisms. To date, there is no evidence of a specific uFFA efflux mechanism in *S. aureus*, and since *SAUSA300_2490* was found through the *in vitro* selection of variants with increased resistance to linoleic acid, I speculated that this gene may contribute to uFFA resistance.

I hereafter refer to *SAUSA300_2490* as *farR* for regulator of fatty acid resistance, and to *SAUSA300_2489* as *farE*, for an effector of fatty acid resistance. Therefore, I hypothesize that *farR* functions to regulate expression of a fatty acid efflux pump encoded by *farE* and that a SNP in *farR* is sufficient to confer resistance to fatty acids. The specific aims of this thesis were to first confirm that the resistance to uFFAs in *S. aureus* is inducible, and examine the role of *farR* and *farE* in this induction. Second, I wanted to examine the nature of regulation that FarR exerts on *farE*, and define the exact operator sites that support FarR binding. Third, I aimed to examine how this novel regulatory system is linked to fatty acid detoxification mechanism both *in vitro* and *in vivo*.

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Chapter 2

2 Inducible expression of a Resistance-Nodulation-Division-Type efflux pump in *Staphylococcus aureus* provides resistance to Linoleic and Arachidonic Acids[1]

2.1 Introduction

Staphylococcus aureus has a dichotomous relation with human hosts, being able to establish an asymptomatic commensal relationship, but also historically known as a leading cause of human infectious morbidity and mortality. Significantly, death attributed to *S. aureus* in the United States is now comparable to mortality rates for AIDS, tuberculosis, and viral hepatitis (1-3). Not surprisingly, therefore, *S. aureus* has been the subject of intensive research on mechanisms of pathogenesis, acquisition and transfer of antibiotic resistance, and efforts to identify potential vaccine antigens (4-6). Until the late 1990's, much of this was directed towards hospital-associated strains of methicillin resistant *S. aureus* (HA-MRSA), to address the anticipated emergence of superbugs that would be resistant to all clinically useful antibiotics (7, 8). However, a new threat emerged in the late 1990's with community acquired MRSA (CA-MRSA). Although these strains evolved in the community setting, one notorious strain known as USA300 has achieved pandemic status across North America, and is now the leading cause of *S. aureus* infections, irrespective of community or hospital origins (9, 10). This has engendered greater attention towards identifying mechanisms of *S. aureus* persistence on human hosts, and host-to-host transmission.

Approximately 25% of humans are persistently colonized by *S. aureus*, where the preferred site of colonization is the anterior nares and, among colonized individuals, the bacterium is also frequently recovered from other body sites, including the axillae, perineum, hands, chest and limbs (11). Accordingly, its ability to persist on skin is an important mediator of transmission, as underscored by the recent discovery that the hyper-transmissible USA300 strain has overcome one of the innate defense barriers of the skin through horizontal gene transfer with *S. epidermidis*, to acquire resistance to toxic polyamines that restrict the growth of other *S. aureus* strains (12, 13). Other innate defence barriers of the skin include its acidic pH, and antimicrobial fatty acids, foremost of which is sapienic acid that is released from triglycerides secreted by the sebaceous glands (14, 15). Nasal secretions also contain antimicrobial fatty acids; primarily linoleic-, arachidonic- and palmitoleic acid, or their corresponding cholesterol esters (16), and infected abscess tissue also contains abundant antimicrobial fatty acids (17, 18).

Consequently, *S. aureus* is exposed to antimicrobial fatty acids, not only during colonization, but also during infection and, as such, it is reasonable to hypothesize that it has evolved mechanisms of intrinsic resistance.

Among mechanisms that have been described, cell surface teichoic acids can selectively restrict the access of palmitoleic acid to the cytoplasmic membrane (19), and a cell surface protein IsdA that is expressed in response to iron-limiting conditions also restricts the access of palmitoleic acid, or its isomer sapienic acid to the cytoplasmic membrane (20). Others have reported that *tet38*, encoding a Major Facilitator Superfamily (MFS) efflux pump, promotes resistance to palmitoleic acid (21). Expression of *tet38* was induced by palmitoleic acid, but not by linoleic acid, which suggested that there could be distinct mechanisms for coping with different antimicrobial fatty acids. Importantly, linoleic acid is an essential fatty acid for humans, which must be obtained from dietary sources, and is an essential precursor for synthesis of arachidonic acid. These two unsaturated fatty acids comprise a major proportion of unsaturated fatty acids in membrane phospholipid (22, 23). Therefore, the ability to sense and respond to linoleic acid could represent a specific sensory mechanism to signal colonization or infection of a human host, and yet specific mechanisms for regulating gene expression and intrinsic resistance in response to linoleic acid have not been reported.

To address this, we drew from our previous observation that exposure of *S. aureus* USA300 to a sub-inhibitory (25 µM) concentration of linoleic acid, caused a robust induction of secreted protease expression, which led to proteolytic processing of a secreted glycerol ester hydrolase, Geh (24). We subsequently noted that when *S. aureus* cultures were supplemented with a trilinolein triglyceride substrate, Geh activity quickly liberated growth inhibitory concentrations of linoleic acid (25). Moreover, 50 µM free linoleic acid imposed a 10-12h growth delay in cultures of *S. aureus* USA300, which was then followed by unimpeded exponential growth, and similar results were obtained with 50 µM trilinolein in wild type *geh* proficient *S. aureus* USA300, whereas growth of a *geh* deficient mutant was unaffected by 50 µM trilinolein (24, 25). From these observations, we hypothesized that, in addition to the induction of expression of secreted proteases, there should also be an inducible mechanism for resistance of *S. aureus* to linoleic acid.

In related studies, selection of *S. aureus* strains that were able to grow at elevated concentrations of glycopeptides led to the identification of point mutations in the *vraS* sensor of antimicrobial glycopeptides (26, 27). Therefore, we adopted a similar strategy, by conducting comparative genome sequencing of USA300 clones that were selected for their ability to initiate growth without a lag phase, when inoculated into media containing 50 µM linoleic acid. We now provide the first description of a novel gene pair *farR-farE* (fatty acid resistance) constituting divergently transcribed genes that, respectively, encode a regulator and effector of *S. aureus* resistance to linoleic and arachidonic acid.

2.2 Materials and methods

2.2.1 Bacterial strains and growth conditions

A list of bacterial strains and plasmids that were used or constructed for this study is provided in Table 2.1. *S. aureus* cultures were maintained as frozen stocks (-80ºC) in 20% glycerol, and streaked on TSB agar when required. TSB was supplemented, when required, with 10 µg/mL of erythromycin or chloramphenicol for propagation of strains bearing resistance markers. *E. coli* strains were grown on LB agar, or LB broth containing 100 µg/mL ampicillin when required. Unless otherwise stated, all cultures were grown at 37°C, and liquid cultures were incubated on an orbital shaking platform at 180 rpm.

For experimental purposes, inoculum cultures of *S. aureus* were prepared by transferring cells, from a single colony, into 13 mL polypropylene tubes containing 3 mL of TSB supplemented with antibiotic, as required, followed by overnight incubation. After determination of optical density at 600 nm (OD_{600}), aliquots of the overnight cultures were diluted into 25 mL of medium in 125 mL flasks, to achieve an initial OD_{600} equivalent to 0.01. To supplement media with different fatty acids, a 5 mM stock concentration was initially prepared in sterile TSB containing 1% DMSO, and then diluted into sterile TSB supplemented with 0.1% DMSO, to achieve the desired concentration of fatty acids, ranging from 5 µM to 100 µM.

Table 2.1 Strains and plasmids used in Chapter 2.

Strain or plasmid	Description	Source or reference
Strains:		
S. aureus:		
USA300 LAC	Community associated MRSA; wild type strain cured of resistance plasmids	(24)
RN4220	$r_K^-\ m_K^+$; capable of accepting foreign DNA	(32)
NE1393	Transposon insertion in SAUSA300_2490; Erm^r	(34)
NE2336	Transposon insertion in SAUSA300_2489; Erm^r	(34)
USA300*farR*::ΦNE	USA300 LAC recipient of transposon from NE1393	This study
USA300*farR*::ΦNE + pLI*farR*	*farR*::ΦNE complemented with native *farR*, cloned in pLI50; Erm^r, Cm^r	This study
USA300*farR*::ΦNE + pCN*farR*	*farR*::ΦNE complemented with pCN*farR* for cadmium inducible expression; Erm^r, Cm^r	This study
USA300farE::ΦNE	USA300 LAC recipient of transposon from NE2336	This study
USA300farE::ΦNE + pLI*farE*	*farE*::ΦNE complemented with native *farE*, cloned in pLI50	This study
USA300farE::ΦNE + pLI50	*USA300farE*::ΦNE with empty pLI50 vector; Cm^r	This study
USA300*Δtet38*	USA300 LAC with internal deletion of *tet38* (SAUSA300_0139)	This study
USA300*Δtet38*-*farE*::ΦNE	USA300*Δtet38* recipient of *farE*::ΦNE transposon insertion; Erm^r	This study

E. coli DH5α	λ⁻ φ80d*lacZ*ΔM15Δ(*lacZYA-argF)U169* *recA1 endA1 hsdr17*(r$_K^-$ m$_K^-$) *supE44 thi-1* *gyrA relA1*	Invitrogen

Plasmids:

pLI50	*E. coli-S. aureus* shuttle vector	(35)
pLI*farR*	pLI50 with native *farR* gene	This study
pLI*farE*	pLI50 with native *farE* gene	This study
pGY*lux*	*E. coli-S. aureus* shuttle vector harboring promoterless *luxABCDE* operon	(37)
pCN51	*E. coli-S. aureus* shuttle vector with P*cad* promoter for cadmium inducible gene expression	(36)
pCN51c	pCN51, with *ermC* cassette replaced by *cat194* cassette from pRN7146	This study
pCN51*farR*	pCN51c with promoterless *farR*, for cadmium inducible expression of *farR*	This study
pKOR-1	*E. coli-S. aureus* shuttle vector; contains P$_{xyl/tetO}$; antisense *secY* RNA expression	(38)
pKOR*Δtet38*	pKOR-1 containing upstream and downstream flanking sequences for deletion of *tet38*	This study

2.2.2 Selection and comparative genome sequencing of fatty acid resistant (FAR) clones

As reported previously, when an overnight culture of *S. aureus* USA300 was inoculated into fresh TSB containing 50 μM linoleic acid, there was a 10-12h lag phase, followed by unimpeded exponential growth (24). Therefore, to promote the selection of fatty acid resistant clones, seven separate flasks of *S. aureus* USA300 were subjected to two consecutive cycles of growth to stationary phase in TSB + 50 μM linoleic acid, after which, samples of each culture were plated for isolation of single colonies. Colonies from each plate were screened to identify fatty acid resistant (FAR) clones that could initiate growth without a lag phase, when inoculated into TSB + 50 μM linoleic acid. A single FAR clone was then selected from each of the seven separate biologic replicates, for comparative genome sequencing. For controls, two single colonies of USA300 were selected after two consecutive cycles of growth in TSB alone.

For comparative genome sequencing, genomic DNA was extracted from *S. aureus* using previously described protocols (28, 29). All samples for comparative genome sequencing were processed at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada; http://www.lrgc.ca) using the Ion Torrent Personal Genome Machine (PGM) (Life Technologies, Carlsbad, CA) and 316 chips. Briefly, genomic DNA was quantified using the Qubit and the Qubit dsDNA HS Assay (Life Technologies, Carlsbad, CA). Samples then underwent fragmentation, adapter and barcode ligation as per the Ion Xpress Fragment Library Kit (4469142 Rev. B), and size selection using the Pippin Prep (Sage Science, Beverly, MA). The size of the final libraries was verified using the Agilent 2100 Bioanalyzer and the High Sensitivity DNA kit (Agilent Technologies Inc., Palo Alto, CA). Barcoded libraries were pooled at equimolar concentrations, based on Qubit values, and the Template Dilution Factor (TDF) for the final pooled library was calculated using molarity determined via qPCR, with the Ion Library Quantification kit (4468802). Diluted libraries were processed as per the Ion OneTouch Template Kit (4468007, Rev. B) for automated clonal amplification, and sequenced using the Ion Express Template 200 kit (4474280), Enrichment Station and the Ion Sequencing 200 Kit (4471999, Rev. B). Sequence reads were mapped to the

genome of *S. aureus* USA300 FPR3757 (30) using CLC Genomics Workbench 7.0 (Boston, MA), and automated detection of single nucleotide polymorphisms (SNPs) was conducted using the Neighborhood Quality Standard algorithm (31).

2.2.3   Strain and plasmid construction

Techniques for genetic manipulation of *S. aureus* were conducted according to established guidelines (32), and as described in our previous work (24, 25, 33). The University of Nebraska transposon mutant library (34) was used as a source of transposon insertions that inactivated SAUSA300_2490 (NE1393) and SAUS300_2489 (NE2336). These were transferred into plasmid cured USA300 strain LAC, creating USA300*farR*::ΦNE and USA300*farE*::ΦNE respectively (Table 2.1). All recombinant plasmids were first constructed as shuttle vectors in *E. coli* DH5α. The integrity of plasmids isolated from *E. coli* were confirmed by restriction enzyme digestion, and nucleotide sequencing of the cloned DNA fragments prior to electroporation into *S. aureus* RN4220 as an intermediate host. From *S. aureus* RN4220, the individual plasmids were then introduced, via electroporation into *S. aureus* USA300 or isogenic derivatives as required. Primers used for PCR amplification of gene segments that were required for plasmid construction are listed in Table 2.2.

Plasmid pLI50 (35) was used to complement mutations in SAUSA300_2490 (*farR*), and SAUSA300_2489 (*farE*). To complement *farE*, a 2.8-kb fragment was amplified by PCR of genomic DNA from *S. aureus* USA300 with forward and reverse primers *farE*_F1 and *farE*_R1. Similarly, a 1.2-kb product containing the native *farR* gene was amplified with primers *farR*_F1 and *farR*-R1. The PCR products were digested with *Kpn*I and *Sac*I, and ligated into pLI50, which had been digested with the same enzymes. To construct pCN51*farR* in which expression of *farR* is dependent on the cadmium inducible P*cad* promoter, we first excised the *ermC* cassette from pCN51 by digestion with *Avr*II and *Xho*I, and replaced it with a 1.0 kb *Avr*II-*Xho*I fragment containing the *cat194* cassette from pRN7146 (36). The resulting pCN51c plasmid was then digested with *Bam*HI and *Asc*I, and ligated to a 605 nt *Bam*HI-*Asc*I fragment containing the promoterless *farR* gene, which was generated by PCR with primers CN*farR*_F and CN*farR*_R. To construct pGY*farE::lux*, in which expression of the luciferase operon is driven from the *farE*

promoter, a 396 bp fragment containing the intergenic segment between SAUSA300_2490 and SAUSA300_2489 (*farE*) was amplified with primers GY*farE*_F and GY*farE*_R, and cloned into the *Bam*HI and *Sal*I sites of pGY*lux* (37).

A markerless in-frame deletion of *tet38* (SAUSA300_0139), encoding a major facilitator efflux pump, was constructed using pKOR-1, following established protocols (25, 38). Briefly, sequences flanking the *tet38* locus were amplified by PCR using primers *tet38* 5′F and *tet38* 5′R to generate the upstream arm, and primers *tet38* 3′F and *tet38* 3′R to generate the downstream arm. The upstream and downstream flanking arms were digested with *Sac*II, ligated to one another, and then recombined into the temperature-sensitive pKOR-1 vector, using *attB1* and *attB2* sites incorporated into the flanking sequences by the respective *tet38* 5′F and *tet38* 3′R primers. The resulting pKOR-1Δ*tet38* vector was first passaged through *S. aureus* RN4220 before being introduced into USA300 by electroporation. The correct deletion of codons 42 through 439 of the *tet38* gene was confirmed by PCR and DNA sequence analysis. The resulting USA300Δ*tet38* strain was then used as a recipient for phage transduction, using USA300*farE*::ΦNE as a donor (Table 2.1), to create USA300Δ*tet38*- *farE*::ΦNE.

Table 2.2 Primers used for construction of plasmids in Chapter 2.

Oligonucleotide	[a]Description
*farE*_F1[b]	cccggtaccCACTTCCATGCAAAAAACCTCC
*farE*_R1[c]	cccgagctcTGTACGGTGTACGAGTGCGTTG
*farR*_F1[b]	cccggtaccTGCAGCTACAATCACTATCCATGC
*farR*_R1[c]	cccgagctcACGGACGCTAAAACAGGTAGTCC
CN*farR*_F[d]	cccggatccgt**taactaa**ttaaCTACACACAAAGGAGAAATGTAG ATG
CN*farR*_R[e]	cccggcgcgccTGAATGTTGGTAACGCTCATGAG
GY*farE*_F[f]	cccggatccTTGTACGGTGTACGAGTGCG
GY*farE*_R[g]	cccgtcgacCGGTGCATTTGTAGCAAGTG
tet38 5′F[h]	*attB1*-GAAACGGTTCTATTGCCAG
tet38 5′R[i]	ggacctccgcggGTTTAAGTCATCAGCAATGGCTACAG
tet38 3′F[i]	ggacctccgcggGTCAGCTTAAATCGTTGGACAC
tet38 3′R[j]	*attB2*-CGCCACCTGATGCTTTTACTTCTAC

[a]Lower case nucleotides indicate 5′-additions, to incorporate restriction endonuclease sites for [b]*KpnI*, [c]*SacI*, [d]*BamHI* and stop codons (bold) in all three reading frames, [e]*AscI*, [f]*BamHI*, [g]*SalI*, [h]*attB1* site GGGGACAAGTTTGTACAAAAAAGCAGGCT for cloning in pKOR-1; [i]*SacII* site; [j]*attB2* site GGGGACCACTTTGTACAAGAAAGCTGGGT for cloning in pKOR-1.

2.2.4 Assay of growth and bactericidal activity

For growth and bactericidal assays, inoculum cultures were supplemented with antibiotic where required, and these cultures were then inoculated into media that lacked antibiotics to assess growth or bactericidal activity, in the presence of antimicrobial fatty acids. For growth assays, flasks containing a 1:5 ratio of flask size to medium volume, and supplemented with the indicated concentrations of fatty acid, were inoculated to an initial OD_{600} of 0.01, and samples were withdrawn at hourly intervals for determination of OD_{600}. All cultures were grown in triplicate, or quadruplicate, as specified in individual Figure Legends. For bactericidal assays, the overnight inoculum cultures were first sub-cultured into 25 mL of fresh TSB alone, to prepare non-induced cells, or TSB containing 20 µM sub-inhibitory fatty acid, to allow for induction of intrinsic resistance mechanisms. After growth to mid-exponential phase (OD_{600} = 0.5), these inoculum cultures were then inoculated into triplicate or quadruplicate flasks of fresh TSB (OD_{600} = 0.01; approximately $2x10^6$ cfu/mL) containing a 100 µM bactericidal concentration of fatty acid. The cultures were then incubated with shaking at 37°C, and aliquots were withdrawn at hourly intervals for preparation of serial dilutions in sterile TSB. Subsequently, 10 µl aliquots from each dilution were spotted in quadruplicate, on to TSB agar plates, and colonies were counted after 24h of incubation. The mean of each quadruplicate technical replicate was entered as a single data point for each flask, from which the mean and standard deviation of the biologic replicate flasks was determined.

2.2.5 Assay for uptake of ^{14}C-linoleic acid

Assays for growth and uptake of ^{14}C-linoleic acid were conducted according to established protocol (39), with modifications, to evaluate the influence of *farE* on accumulation of ^{14}C-linoleic acid in *S. aureus* cells. Briefly, quadruplicate cultures of *S. aureus* USA300 or isogenic USA300*farE*::ΦNE complemented with empty pLI50 vector, or pLI*farE*, were grown in TSB + 20 µM linoleic acid to an OD_{600} of approximately 0.3, to allow for induction of *farE*. The cultures were then supplemented with an additional 50 µM dose of linoleic acid and returned to the shaker. After 30 minutes of exposure to 50 µM linoleic acid, aliquots were withdrawn, and supplemented with 0.2 µCi/mL of ^{14}C-linoleic acid. Aliquots of 200 µL were then removed at intervals of 1, 2, 5, and 10

minutes, and samples from each replicate were simultaneously filtered onto 0.45 µM membrane filter discs using a vacuum manifold. The filters were then washed twice with 4 mL of 0.1 M phosphate buffer, pH 7.0 containing 1% Triton X-100, and after drying, were placed in scintillation vials containing 4 mL of Cytoscint scintillation cocktail (Fisher Scientific). Accumulated ^{14}C-linoleic acid was then quantified using a Beckman LS 6500 scintillation system. Data are expressed as pMol of ^{14}C-linoleic acid accumulated, per µg of total cell lysate protein in each sample.

2.2.6   *farE::lux* reporter gene assays

Inoculum cultures harboring of pGY*farE*::*lux* or pGY*lux* control plasmid were subcultured into triplicate or quadruplicate flasks of TSB, or TSB supplemented with different fatty acids, to achieve an initial OD_{600} = 0.01. The cultures were incubated at 37°C with orbital shaking, and samples were withdrawn at hourly intervals for OD_{600} determinations. For quantification of luminescence, 4 x 200-µL aliquots of each sample were added to 96 well white opaque flat bottom plates (Greiner bio-one). After supplementing each well with 20 µL of 0.1% vol/vol decanal in 40% ethanol, luminescence measurements were immediately taken on a BioTek Synergy H4 Hybrid Reader (BioTek; Winooski, VT) with 1 second of integration and a gain of 200. Data values were recorded as relative light units (RLU), corrected for background by subtraction of values recorded from cultures harbouring the empty pGY*lux* vector. The data points were standardized for differences in growth by dividing RLU values with the recorded OD_{600} of the cultures, when samples were withdrawn.

2.2.7   Data analyses

Data points for growth, viability, and luciferase reporter gene assays were plotted and analyzed using Graph Pad Prism v6.0f. Significant differences at specific time points were determined by unpaired one-tailed Student's t-tests.

2.3 Results

2.3.1 Identification of single nucleotide polymorphism in linoleic acid resistant variants of *S. aureus*

The preferred site of *S. aureus* colonization of humans is the anterior nares, where concentrations of linoleic acid in nasal secretions can reach 40- to 50 µM (16). These values correlate with our previous work, where 50 µM linoleic acid caused a 10-12h lag phase in growth of USA300, followed by unimpeded exponential growth (24). Following up on this, we observed that when stationary phase cells from a primary culture grown in TSB + 50 µM linoleic acid were re-inoculated into the same medium, growth resumed without a lag phase (Appendix 2). To determine if this was due to the selection of genetic variants with increased resistance to linoleic acid, stationary phase cells from this second culture were plated on TSB agar for selection of single colonies. From these, we identified several that could initiate growth without a lag phase, when inoculated into TSB + 50 µM linoleic acid. Seven such fatty acid resistant (FAR) clones were subjected to comparative genome sequencing, and two of these, designated FAR6 and FAR7, had an identical single nucleotide polymorphism (SNP); a C>T transition that alters the H_{121} codon (CAT) to Y (TAT) in a putative transcriptional regulator encoded by SAUSA300_2490 (30). FAR6 had a second SNP in a pyruvate oxidase encoded by *cidC*. Therefore, we focused on FAR7, which had just one SNP in SAUSA300_2490, and re-sequencing of this gene in USA300 and FAR7 confirmed the unique SNP in FAR7.

2.3.2 Description of the *farE-farR* locus

We hypothesize that SAUSA300_2490, and a divergently transcribed gene SAUSA300_2489, respectively comprise a regulator and effector gene pair that we have designated as *farR* and *farE*, to denote predicted functions as a <u>r</u>egulator and <u>e</u>ffector of <u>f</u>atty <u>a</u>cid <u>r</u>esistance. These assignments are supported by bioinformatics analyses. *farR* encodes a 182 amino acid protein, with an N-terminal TetR family DNA binding domain (2.33e^{-04}), and overall similarity to the AcrR cluster of orthologous groups of proteins (6.52e^{-09}). In Gram-negative bacteria, AcrR regulators control expression of efflux pumps belonging to the AcrB family, which are often encoded by divergently transcribed genes, as with *acrR-acrABC* in *E. coli* (40) and orthologous *mtrR-mtrCDE* arrangement in *N.*

gonorrhoeae (41). Similarly, *farE* is divergently transcribed from *farR* and encodes an 822 amino acid protein that is annotated as a drug exporter of the resistance-nodulation-division (RND) superfamily (30), to which AcrB and orthologous efflux pumps are also assigned (42). Genome annotation also assigns FarE to the MMPL family of proteins, on the basis of homology to large membrane proteins of *Mycobacterium tuberculosis*, that transport mycolic acids to the cell surface (43). Using protein structural modeling programs HHPRED and PHYRE2 (44, 45), FarR was predicted with greater than 99% confidence, to resemble known AcrR family regulators, including PfmR and FadR of *Thermus thermophilus*, which control expression of genes involved in fatty acid synthesis and metabolism (46, 47), and MtrR; an efflux pump regulator of *Neisseria gonorrhoeae* (41, 48). Likewise, 80% of the FarE amino acid sequence was modeled with 100% confidence, on the structure of AcrB from *E. coli* (49).

2.3.3   *farR* is required for inducible resistance to linoleic acid

We hypothesized that *farR* should regulate expression of *farE* in response to antimicrobial fatty acids, which was addressed by constructing a *farE::lux* reporter, where expression of the *lux* operon is under transcriptional control of the *farE* promoter. When USA300-pGY*farE::lux* was cultured in TSB, there was a modest peak of luciferase activity in early exponential growth, which quickly dissipated (Fig. 2.1). However, in TSB + 20 μM linoleic acid, luciferase activity was strongly induced in early exponential phase cells, and again dissipated as the cells progressed towards stationary phase. Importantly, no induction was observed in USA300*farR*::ΦNE cells. Although USA300*farR* appeared to exhibit superior growth to wild type USA300 in TSB + 20 μM linoleic acid (Fig. 2.1), our further analysis on this phenomenon uncovered that it reflects a growth penalty that is imposed on USA300 by forced expression of the *luxABCDE* genes. This was evident from a growth comparison of USA300 harboring either pGY*farE::lux* or empty pGY*lux*, in TSB + 20 μM linoleic acid, where cells carrying pGY*farE::lux* exhibited significantly slower growth compared to USA300 with empty vector (Appendix 3).

These assays suggested that USA300 should exhibit inducible resistance to the antimicrobial activity of linoleic acid. It was previously reported that exponential phase

cells of *S. aureus* were significantly more sensitive to the bactericidal activity of antimicrobial fatty acids, relative to stationary phase cells (17), which we confirmed in a preliminary experiment (Appendix 4). Therefore, to assess inducible resistance, USA300 and USA300*farR*::ΦNE were grown to mid-exponential phase in TSB (non-induced), or TSB + 20 μM linoleic acid (induced), and then diluted to 10^6 cfu/mL in fresh TSB containing 100 μM linoleic acid. Non-induced USA300 suffered a > 3-log loss of viability after 1h of exposure to 100 μM linoleic acid (Fig. 2.2A), while the induced cells retained significantly greater viability at all time points, such that there was only an approximate 40-fold loss of viability after 5h. Furthermore, the induced USA300*farR*::ΦNE cells exhibited a significantly greater loss of viability compared to induced USA300, after 2h and onwards. Although the induced USA300*farR*::ΦNE cells initially retained significantly greater viability compared to non-induced USA300, they exhibited a progressive loss of viability, such that after 4h of exposure, the remaining viable cells did not significantly differ from non-induced USA300 cells.
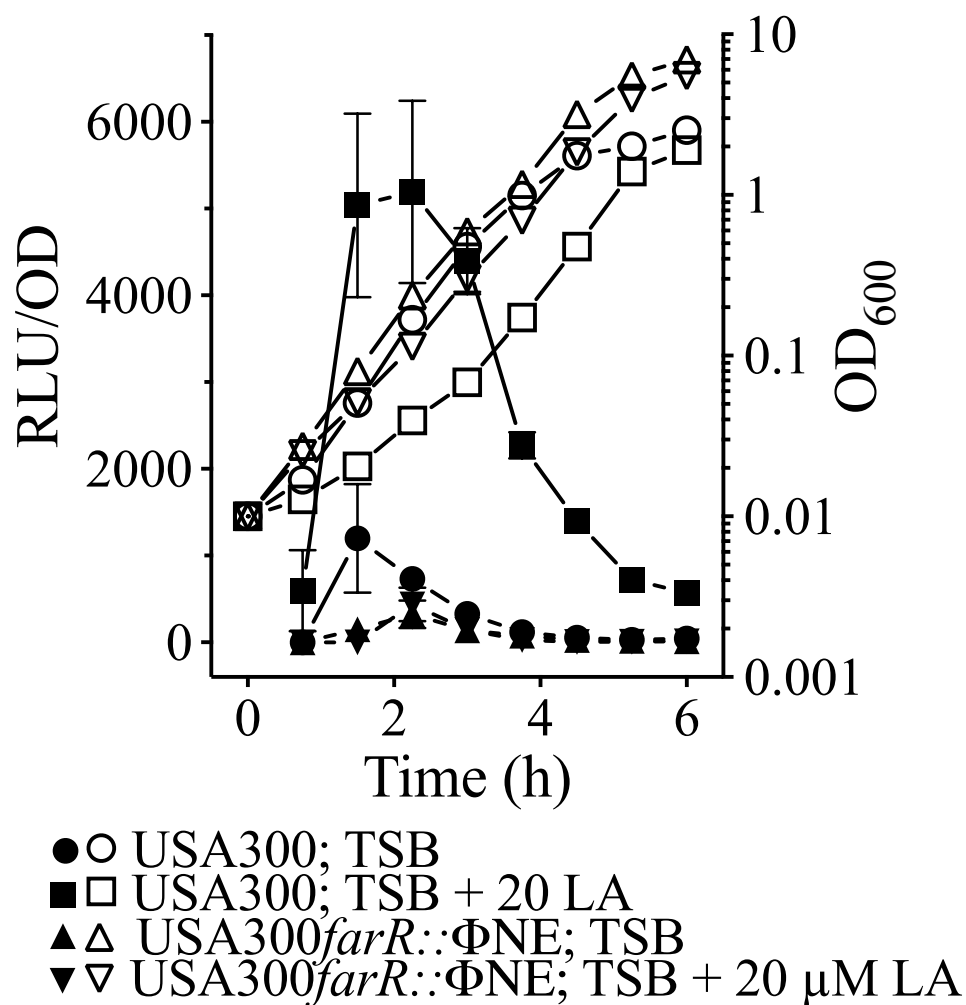
Figure 2.1 Linoleic acid induces expression of *farE*. Growth (OD$_{600}$; open symbols) and relative luminescence units (RLU/OD; closed symbols) of USA300 and USA300*farR*::ΦNE, harboring the pGY*farE::lux* reporter vector, are charted. USA300 was grown in TSB or TSB + 20 μM linoleic acid (LA); USA300*farR*::ΦNE was grown in TSB or TSB + 20 μM linoleic acid. Each value represents the mean and standard deviation of three separate cultures, and each culture was subjected to quadruplicate luminescence readings at each time point.

Viability in 100 μM linoleic acid
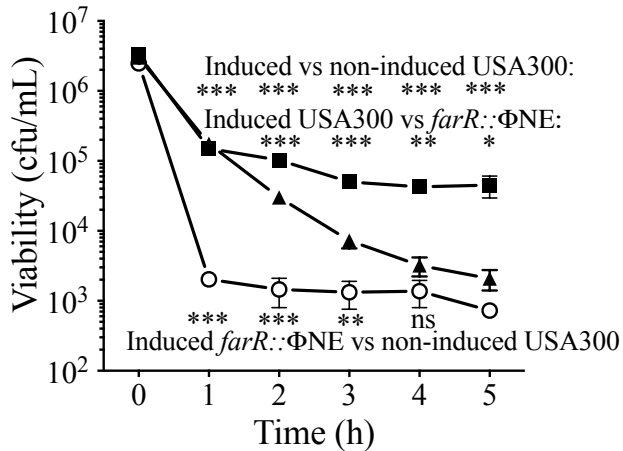
A.

B.
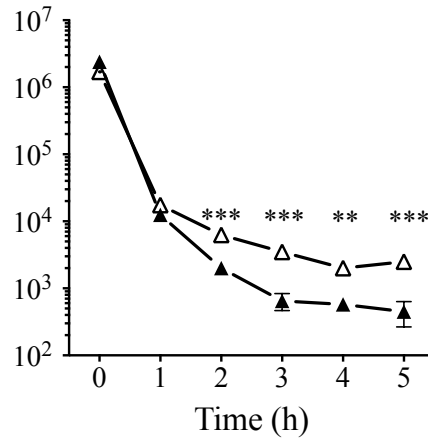
Figure 2.2 Sensitivity of USA300 and USA300*farR*::ΦNE to the bactericidal activity of 100 μM linoleic acid. A. USA300 or USA300*farR*::ΦNE challenge cells were grown to mid-exponential phase in TSB or TSB + 20 μM linoleic acid and then diluted to 10^6 cfu/mL in TSB containing 100 μM linoleic acid, followed by monitoring of viability at hourly intervals. **B.** USA300*farR*::ΦNE was complemented with empty pLI50 vector or pLI*farR* and assayed for viability in 100 μM linoleic acid, after initial growth in TSB + 20 μM linoleic aicd. All data points represent the mean ± standard deviation, of viability determinations from quadruplicate cultures. Significant differences in viability at each time point were determined by unpaired one-tailed Student's t-test; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, nonsignificant.

To validate a role for *farR*, USA300*farR*::ΦNE was complemented with empty pLI50, or pLI*farR* harboring *farR* and its native promoter, to determine whether pLI*farR* could restore inducible resistance. Accordingly, when pre-induced by growth in 20 μM linoleic acid, USA300*farR::*ΦNE + pLI*farR* retained significantly greater viability after 2h of exposure to 100 μM linoleic acid, compared to USA300*farR::*ΦNE + pLI50 (Fig. 2.2B). Nevertheless, pLI*farR* did not appear to restore the level of inducible resistance to that of wild type USA300, which retained approximately 10^5 cfu/mL viable cells after 5h of exposure (Fig. 2.2A). We reasoned that this could be due to two variables; first, that *farR* might be expressed at a high level from its native promoter on a multi-copy plasmid, and second, that the FarR protein could engage nucleotide sequences on pLI*farR*, which contained the entire *farE-farR* intergenic segment, and these *in trans* interactions could limit the ability of FarR to regulate *farE* on the chromosome. To overcome these limitations, we expressed *farR* using the cadmium inducible P_{cad} promoter, and observed an approximate 100-fold difference in viability when USA300*farR::*ΦNE + pCN*farR* cells were exposed to 100 μM linoleic acid, in the presence or absence of 10 μM cadmium (Appendix 5). Cumulatively, these data support the contention that *farR* is required to manifest an inducible resistance phenotype in *S. aureus* USA300.

2.3.4 *farE* contributes to persistence and growth of *S. aureus* in the presence of linoleic acid

We previously established that USA300 could grow in TSB containing 25 μM linoleic acid, whereas 50 μM linoleic acid imposed a 10-12h lag phase. Our current reporter gene assays also established that *farE* was induced by growth in TSB containing 20 μM linoleic acid. Therefore, we expected that *farE* would be required to support growth of *S. aureus* USA300 at a 25 μM upper threshold of linoleic acid, and that induction of *farE* would confer protection against challenge of *S. aureus* with a 100 μM bactericidal concentration. To address the growth requirement, USA300 or USA300*farE*::ΦNE were cultured in TSB containing 5, 10, 20, or 25 μM linoleic acid. USA300 was not adversely affected by 5- or 10 μM linoleic acid, but exhibited slower growth in 20- or 25 μM linoleic acid (Fig. 2.3A). Comparatively, USA300*farE*::ΦNE exhibited similar behavior at 5, 10 and 20 μM linoleic acid, but was unable to initiate growth over an 8h incubation

in 25 µM linoleic acid (Fig. 2.3B). Furthermore, when USA300*farE*::ΦNE was complemented with pLI*farE*, we observed growth restoration not only in 25 µM linoleic acid (Fig. 2.3B), but also in up to 100 µM linoleic acid (Fig. 2.3C); compare this to wild type USA300, which was unable to grow in 50 µM linoleic acid (Fig. 2.3C).

To ensure that the role of *farE* was not dependent on factors that are uniquely associated with the CA-MRSA strain USA300 genetic background, we transduced *farE*::ΦNE into *S. aureus* SH1000, which is a methicillin susceptible laboratory strain that has the same multi-locus sequence type (MLST) as USA300 (50). Although SH1000 exhibited somewhat greater intrinsic resistance to linoleic acid, as evident from its ability to grow in TSB + 50 µM linoleic acid, SH1000*farE*::ΦNE exhibited an extended lag phase, with no obvious growth over 6h (Fig. 2.3D). Therefore, *farE* promotes growth of both MRSA and MSSA strains at elevated concentrations of linoleic acid.

To evaluate the role of *farE* in promoting inducible resistance, USA300 and USA300*farE*::ΦNE were grown in TSB alone, or TSB containing 20 µM linoleic acid, prior to subculture into 100 µM linoleic acid (Fig. 2.4). Consistent with *farE* not being appreciably expressed in non-induced cells, the non-induced USA300 and USA300*farE*::ΦNE cultures both suffered a rapid loss of viability on exposure to 100 µM linoleic acid. However, when the cells were grown under inducing conditions prior to challenge with 100 µM linoleic acid, USA300 exhibited only a 10- to 40-fold loss of viability over 5h, and retained significantly greater viability at all time points, compared to USA300*farE*::ΦNE. Interestingly, the induced USA300*farE*::ΦNE challenge cells still retained significantly greater viability compared to non-induced USA300, which suggests that factors in addition to *farE* may also promote inducible resistance. Cumulatively, these data confirm that *farE* contributes to the inducible resistance of *S. aureus* to the bactericidal activity of 100 µM linoleic acid, and is also required to support growth in as low as 25 µM linoleic acid. It further appears that resistance is proportional to *farE* expression, as suggested by the ability of pLI*farE* to support growth of USA300*farE*::ΦNE at concentrations of linoleic acid that could not be tolerated by USA300 (Fig. 2.3C).

A. USA300

B. USA300*farE*::ΦNE

C. USA300 and USA300*farE*::ΦNE

D. SH1000 and SH100*farE*::ΦNE
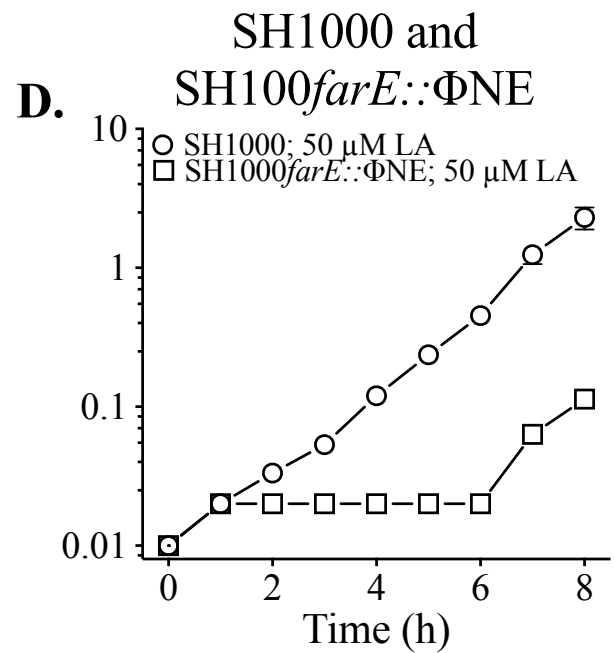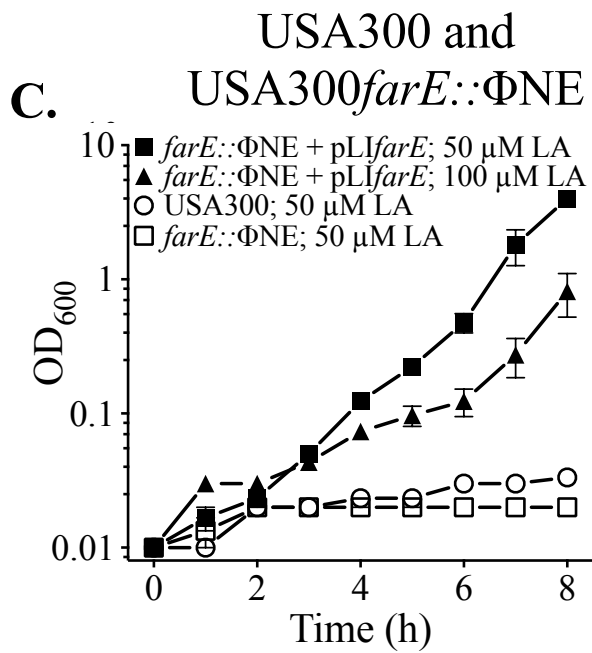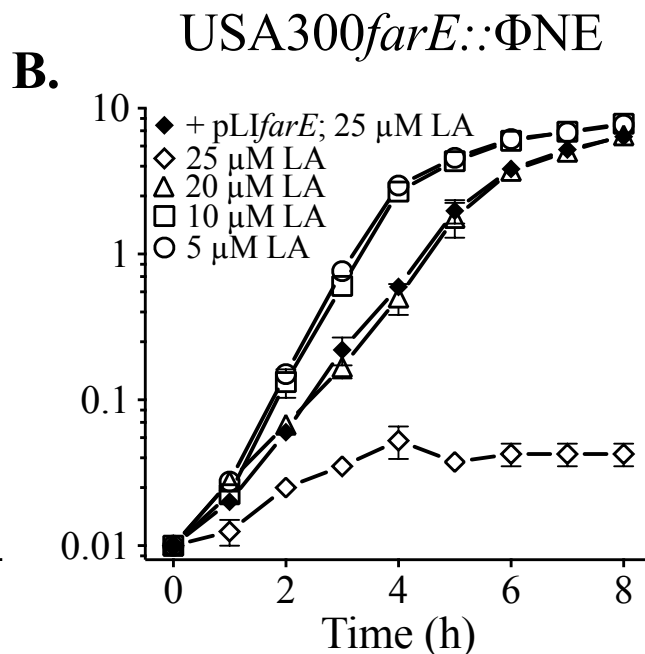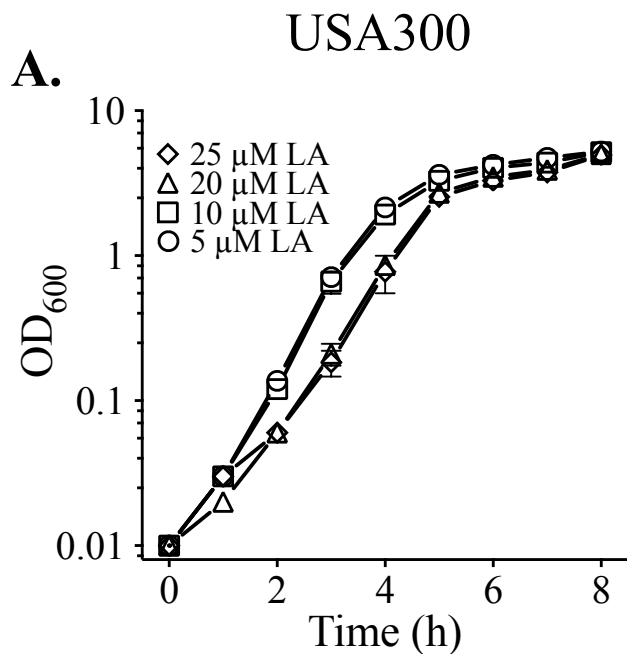
Figure 2.3 Mutation of *farE*::ΦNE enhances sensitivity of *S. aureus* to toxicity of linoleic acid. Growth of USA300 **(A)** or USA300*farE*::ΦNE **(B)** in TSB supplemented with 5 µM, 10 µM, 20 µM or 25 µM linoleic acid, and that of USA300*farE*::ΦNE + pLI*farE* in TSB + 25 µM linoleic acid. **C.** Growth of USA300 or USA300*farE*::ΦNE in TSB + 50 µM linoleic acid and growth of USA300*farE*::ΦNE + pLI*farE* in 50 µM or 100 µM linoleic acid. **D.** Growth of *S. aureus* SH1000 or SH1000 *farE*::ΦNE in TSB + 50 µM linoleic acid. Each data point represents the mean of triplicate (**A**, **C** and **D**) or quadruplicate (**B**) cultures.
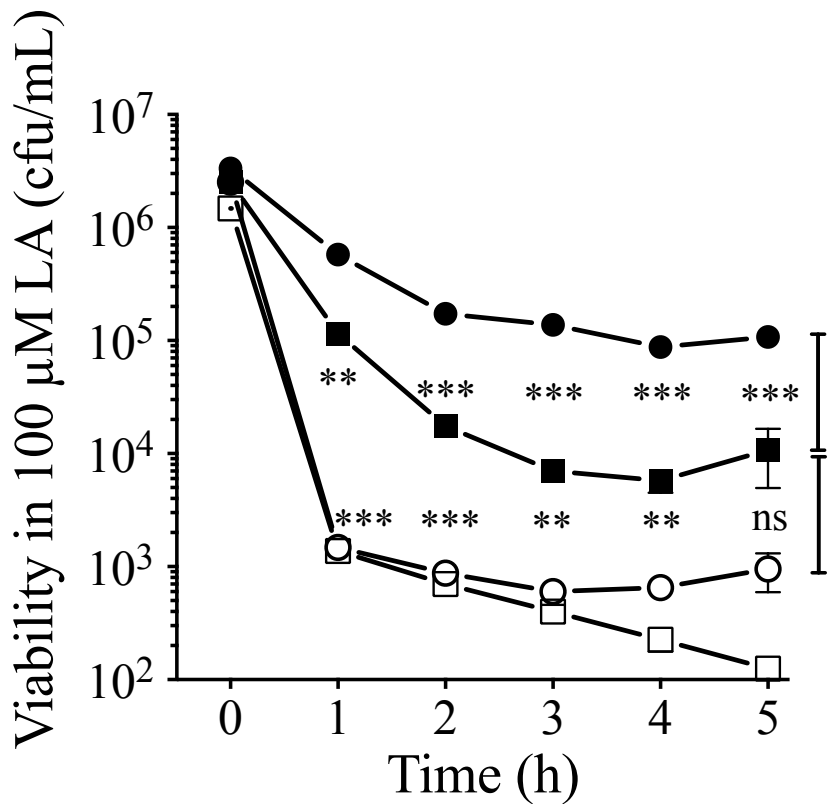
Figure 2.4 Sensitivity of USA300 and USA300*farE*::ΦNE **cells to the bactericidal activity of 100 μM linoleic acid.** Cells of USA300 or USA300*farE*::ΦNE were exposed to 100 μM linoleic acid after growth to mid-exponential phase in TSB or TSB + 20 μM linoleic acid. Each data point represents the mean of quadruplicate cultures. *P* values are indicated by asterisks where **, *P* < 0.01; ***, *P* < 0.001; ns, non-significant.

2.3.5   The FAR7 clone exhibits increased expression of *farE*

FAR7 is distinguished from USA300 by a SNP in *farR* that changes H_{121} to Y in the gene product. This clone was selected for its ability to grow without a lag phase in TSB + 50 μM linoleic acid, and our data suggest that this should be due to increased expression of *farE*, as a consequence of the SNP in *farR*. This was confirmed by conducting *farE::lux* reporter gene assays, in both USA300 and FAR7 (Fig. 2.5). When grown in TSB, FAR7 exhibited significantly greater luciferase activity compared to USA300, and during growth in TSB + 20 μM linoleic acid, the luciferase activity in FAR7 significantly exceeded that of USA300. Therefore, the SNP that causes a $H_{121}Y$ substitution in FarR results in a constitutive level of *farE* expression during growth in TSB, and permits a significantly greater induced level of expression, than could otherwise be achieved in USA300.

2.3.6   An $H_{121}Y$ substitution in FarR is sufficient for increased resistance to linoleic acid

Since USA300 and FAR7 are differentiated on the basis of a SNP that causes a $H_{121}Y$ substitution in FarR, we expected that this alone would be sufficient to promote increased resistance to linoleic acid. Accordingly, although FAR7 and USA300 exhibited no difference in growth when cultured in TSB, FAR7 was uniquely able to grow in TSB + 100 μM linoleic acid (Fig. 2.6A). In bactericidal assays, non-induced USA300 and FAR7 both suffered a similar rapid loss of viability when exposed to 100 μM linoleic acid (Fig. 2.6B). Therefore, although there is some constitutive expression of *farE* during growth of FAR7 in TSB, this is not sufficient to promote resistance to 100 μM linoleic acid. However, when the assay was conducted with cells grown under inducing conditions, FAR7 did not exhibit any significant loss of viability over 5h of exposure to 100 μM linoleic acid, and exhibited significantly greater retention of viability from 3- to 5h, compared to USA300 (Fig. 2.6B). These observations are consistent with our *farE::lux* assays, where FAR7 exhibited a significantly higher induced level of *farE* expression compared to USA300, and support the contention that increased expression of *farE* correlates with increased resistance.
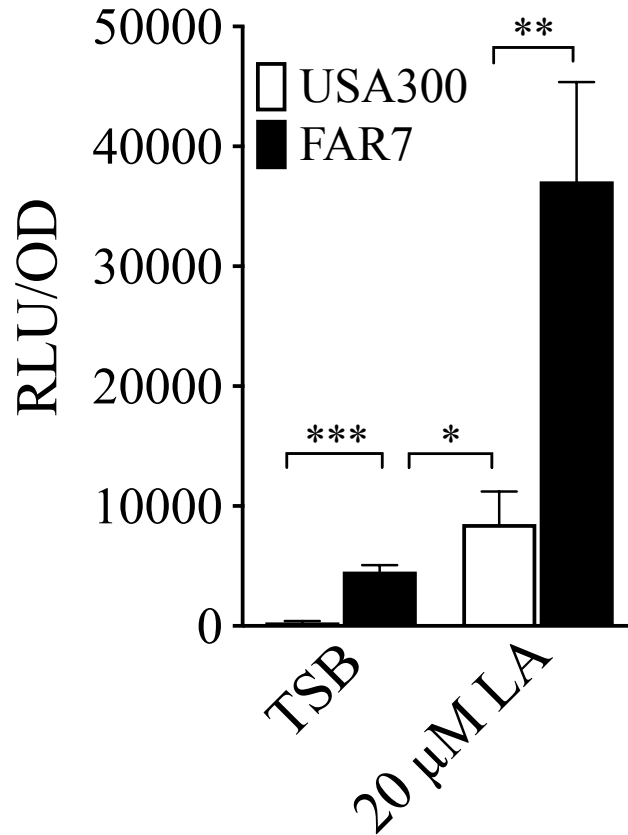
Figure 2.5 The FAR7 SNP causes enhanced induction of *farE* expression. The cultures were grown in TSB or TSB + 20 μM linoleic acid as indicated. Data are expressed as relative luminosity units (RLU), standardized to one OD_{600} unit. Values represent the mean of four replicates from each of four independent cultures. Measurements were taken from triplicate cultures when OD_{600} values reached approximately 0.5, and P values are indicated by asterisks where *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
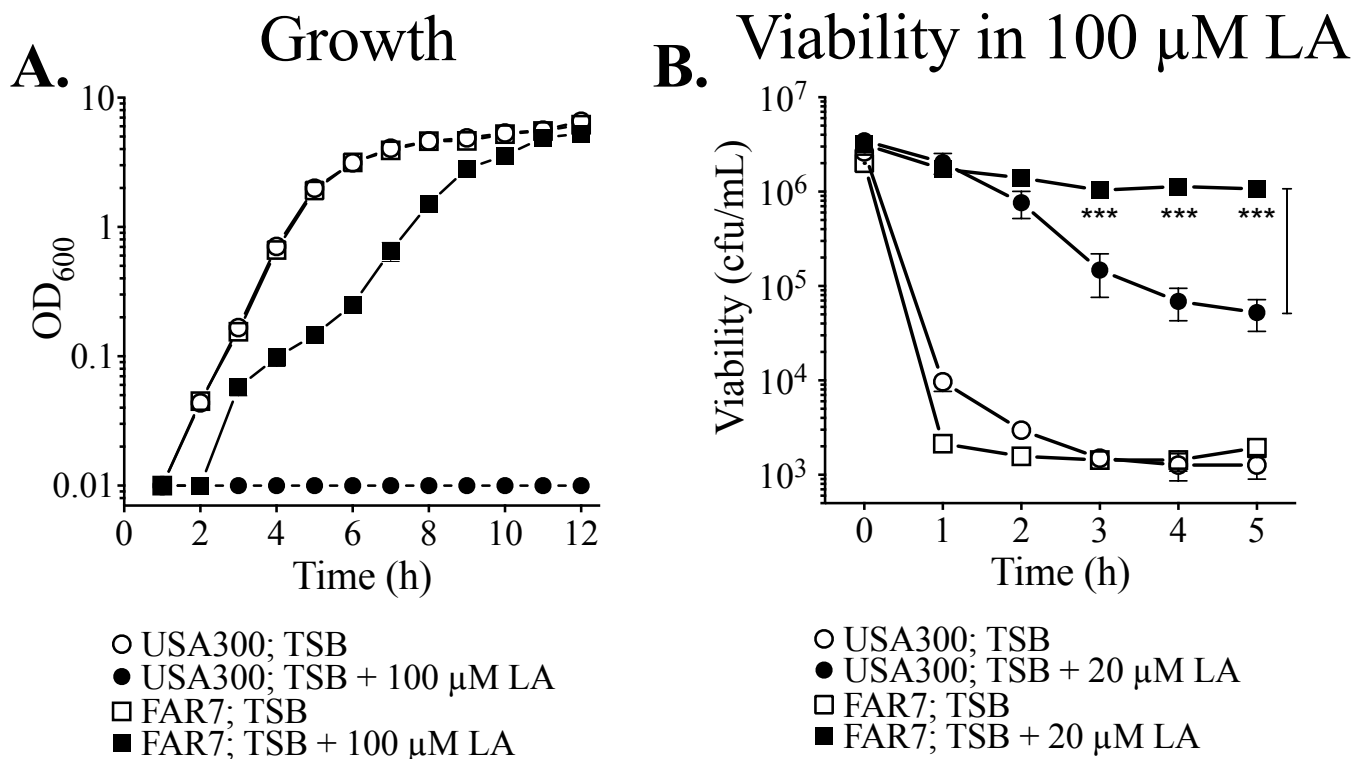
Figure 2.6 FAR7 is more resistant than USA300 to linoleic acid. A. Growth analysis of USA300 and FAR7 cultured in TSB or TSB + 100 μM linoleic acid. **B.** Bactericidal activity of 100 μM linoleic acid measured with USA300 or FAR7 challenge cells, prepared by growth to mid-exponential phase in TSB or TSB + 100 μM linoleic acid. Each data point represents the mean of triplicate cultures. *P* values for comparison of induced USA300 and induced FAR7 cells are indicated by asterisks where ***, *P* < 0.001.

To further define the impact of the $H_{121}Y$ substitution, USA300*farR*::ΦNE was transformed with pLI50 harboring wild type *farR*, or the variant *farR7* allele derived from FAR7. With no complementation, USA300*farR*::ΦNE exhibited no growth over an 8h incubation in TSB + 50 μM linoleic acid, and cells complemented with wild type pLI*farR* were also unable to grow (Fig. 2.7). However, cells complemented with the variant *farR7* allele acquired the ability to grow in 50 μM linoleic acid, and also at a reduced rate in 100 μM linoleic acid. Therefore, a SNP that introduces a $H_{121}Y$ substitution in FarR is alone sufficient to confer increased resistance of *S. aureus* towards linoleic acid, presumably due to increased expression of *farE*.

2.3.7   Role of *farE* in resistance to other uFFA

Although *farE* is induced by and promotes resistance to linoleic acid, *S. aureus* would be exposed to a varying diversity and abundance of free fatty acids, dependent on the context within the human body. In a tissue abscess, pus contains high concentrations of unsaturated free fatty acids, which could be derived from triglyceride (18, 51), or human cell membrane phospholipid, where the major unsaturated fatty acids, oleic (C18:1), linoleic (C18:2), and arachidonic acid (C20:4), each comprise approximately 13- to 15% of the total fatty acid content (22, 23). Conversely, although sapienic acid or its isomer palmitoleic acid (C16:1) do not comprise a major proportion of the fatty acid profile of phospholipid, sapienic acid is the major unsaturated fatty acid in human sebum, both as free fatty acid, and in sebum triglyceride (14, 52). Therefore, to better understand the biological role of *farE*, we evaluated the specificity of *farE* induction by these different fatty acids, and the extent to which *farE* confers resistance to other fatty acids.

To evaluate the specificity of induction, USA300-pGY*farE::lux* was grown to an OD_{600} of ~ 0.5 in TSB, or TSB supplemented with 20 μM fatty acid, followed by assay of luciferase activity (Fig. 2.8A). There were significant differences in the ability of different 18-carbon chain length fatty acids to induce *farE::lux*, such that no induction was observed with saturated stearic acid (C18:0), or oleic acid (C18:1), while linoleic acid (C18:2) was a strong inducer. Strikingly, arachidonic acid (C20:4) promoted a significantly higher level of expression compared to linoleic acid, while linolenic acid (C18:3), together with palmitoleic acid (C16:1) and its isomer sapienic acid, each

facilitated an intermediate level of expression, which was significantly greater than that of TSB alone, but significantly less than that of linoleic and arachidonic acid.

Consistent with the modest induction by 20 µM palmitoleic acid, when a bactericidal assay was conducted with USA300 and USA300*farE::*ΦNE cells that were pre-induced by growth in 20 µM palmitoleic acid, there were no significant differences in retention of viability after exposure to 100 µM palmitoleic acid (Fig. 2.8B). However, when this assay was performed with arachidonic acid, USA300 retained significantly greater viability after 2h of exposure, compared to USA300*farE::*ΦNE (Fig. 2.8B). Therefore, *farE* appears to have a primary role in mediating resistance to linoleic- and arachidonic acid, which are the most effective inducers of *farE* expression.

Although *farE* did not promote resistance to palmitoleic acid, we nevertheless observed a significant induction of expression by 20 µM palmitoleic acid (Fig. 2.8A), in addition to which, FAR7 was able to grow in TSB containing 50 µM palmitoleic acid, whereas USA300 could not (Appendix 6). This suggested that *farE* could still promote resistance to palmitoleic acid, if expressed at a sufficiently high level. Furthermore, it was recently reported that *tet38*, which encodes a major facilitator superfamily efflux pump, was induced by palmitoleic acid and contributed to resistance (21). Therefore, we considered that one efflux pump might compensate for loss of another, which could obfuscate the phenotype of USA300*farE::*ΦNE when tested with palmitoleic acid. To address this, we constructed a markerless *Δtet38* mutation in USA300, which was assayed for growth in TSB + 25 µM or 40 µM palmitoleic acid. The higher concentration imposed a slower growth rate, as evident from a time of 5h being required for USA300 to achieve an OD_{600} = 0.5, compared to approximately 3h in 25 µM palmitoleic acid (Fig. 2.9). Nevertheless, there were no discernible differences in growth between USA300, and the individual USA300*Δtet38* or USA300 *farE::*ΦNE mutants, or combined USA300*Δtet38-farE::*ΦNE double mutant. Therefore, neither *farE* nor *tet38* exerted a significant impact on resistance to palmitoleic acid under the conditions that we have tested.
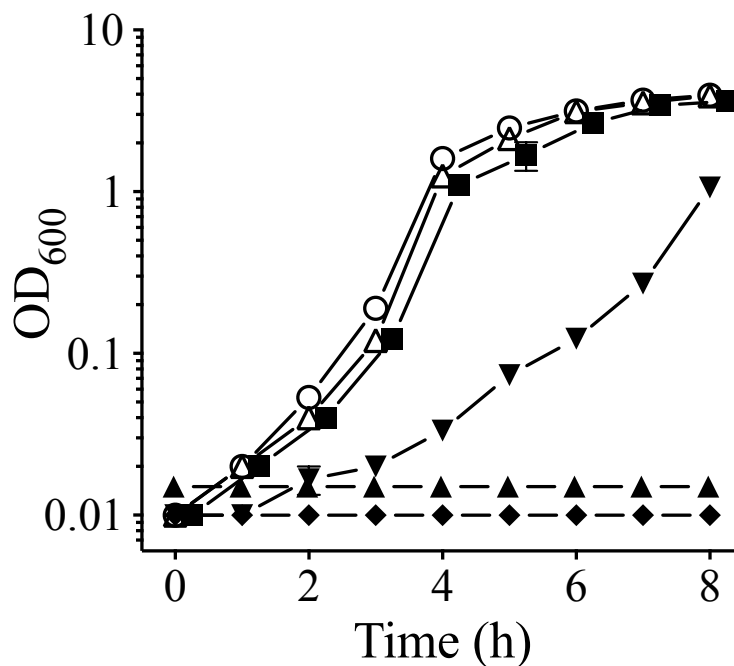
Figure 2.7 The variant *farR7* allele, but not wild type *farR*, enables the ability of USA300*farR*::ΦNE to grow at inhibitory concentrations of linoleic acid. USA300 was grown in TSB + 25 μM linoleic acid, USA300*farR*::ΦNE was grown in 25 μM or 50 μM linoleic acid, USA300*farR*::ΦNE + pLI*farR* was grown in 50 μM linoleic acid, and USA300*farR*::ΦNE + pLI*farR7* was grown in 50 μM or 100 μM linoleic acid. All data points represent the mean of triplicate cultures.

A. *farE::lux* Activity

B. Viability after 3h exposure to 100 μM fatty acid
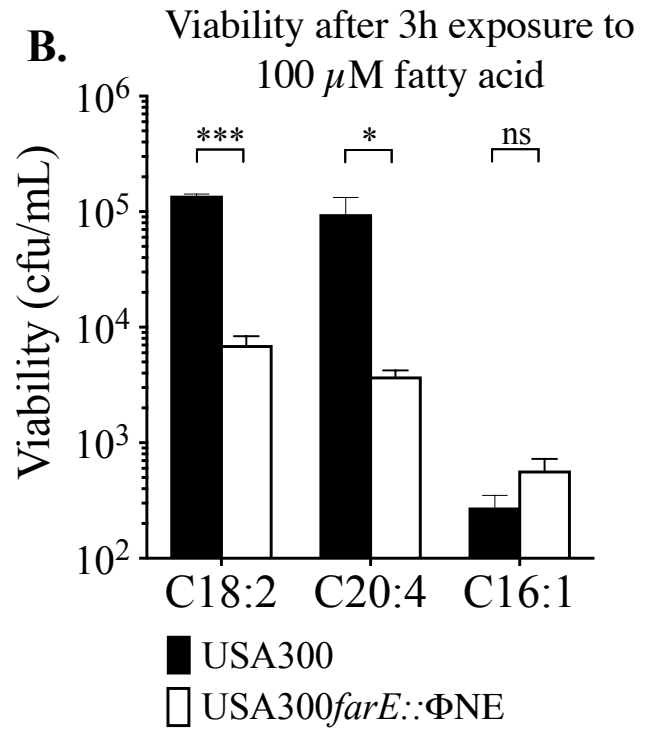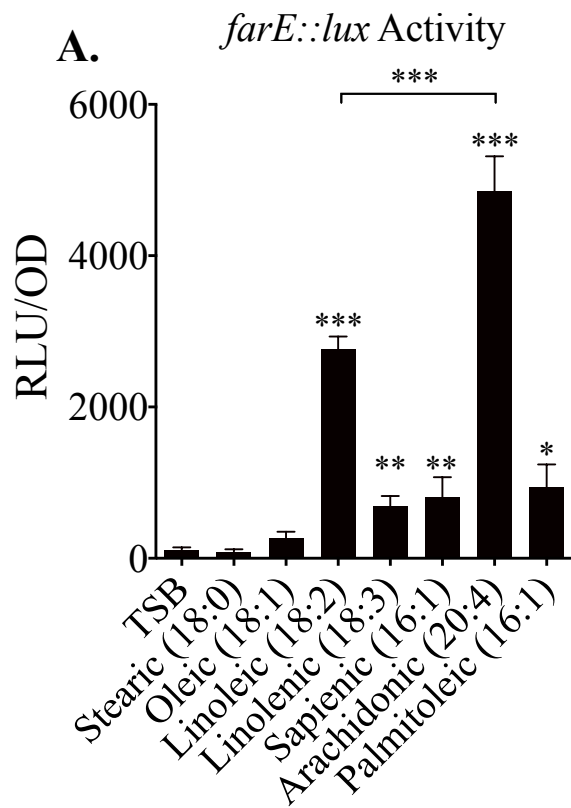
Figure 2.8 Influence of different antimicrobial fatty acids on induction of *farE*, or viability of *S. aureus* USA300 and USA300*farE*::ΦNE. A. Quantification of pGY*lux::farE* dependent luciferase activity in *S. aureus* USA300 grown to OD_{600} = 0.5 in TSB alone, or TSB supplemented with 20 µM of fatty acid, as indicated. Each value represents the mean of quadruplicate measurements, from each of four replicate cultures. *P* values indicate significant differences compared to growth in TSB alone, or significant difference between linoleic and arachidonic acid. **B.** Bactericidal activity of 100 µM linoleic acid (C18:2), arachidonic acid (C20:4), or palmitoleic acid (C16:1) towards USA300 or USA300*farE*::ΦNE cells. The inoculum cultures were grown to OD_{600} = 0.5 in TSB supplemented with 20 µM of the respective fatty acids, prior to challenge with a 100 µM bactericidal concentration. Asterisks indicate *P*-values of significant differences between USA300 and USA300*farE*::ΦNE. Each value represents the mean of viability determination from quadruplicate cultures. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant.
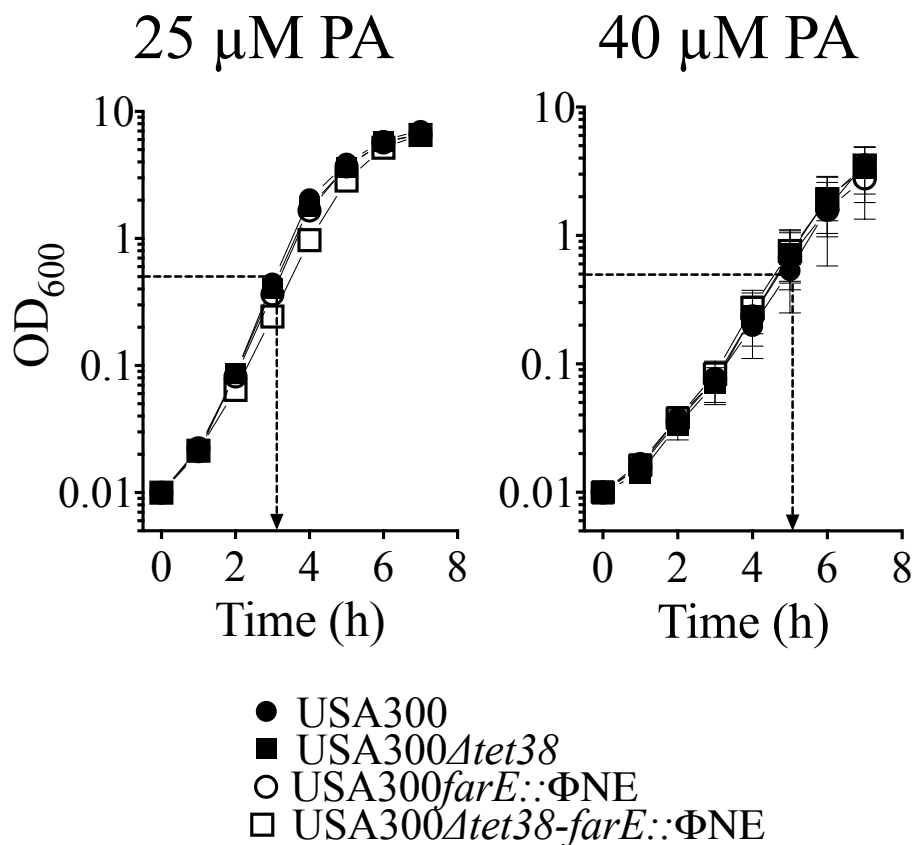
Figure 2.9 Effect of *farE*::ΦNE and *Δtet38* mutations on growth of *S. aureus* in the presence of 25 μM or 40 μM palmitoleic acid (PA). USA300, USA300*farE*::ΦNE, USA300*Δtet38,* and USA300*Δtet38-farE*::ΦNE were grown in TSB supplemented with 25 μM or 40 μM palmitoleic acid (PA), as indicated. The dotted line with arrow depicts the time of growth at which $OD_{600} = 0.5$.

2.3.8   Inactivation of *farE* promotes increased uptake of ^{14}C-linoleic acid

Although many bacteria can derive energy from exogenous fatty acids through an inducible β-oxidation pathway (53), *S. aureus* lacks this ability, and its primary means of coping with exogenous fatty acids is through incorporation into phospholipid (19, 39, 54, 55). Since our data suggest that FarE promotes efflux of fatty acids, we expected that inactivation of *farE* would promote increased uptake of exogenous fatty acid. Prior to quantifying uptake of ^{14}C-linoleic acid, we first conducted a mock assay, to evaluate the ability USA300 and USA300*farE::*ΦNE to recover from exposure to an abrupt increase in the concentration of linoleic acid. Cultures were grown to OD_{600} = 0.3 in TSB supplemented with sub-inhibitory 20 μM linoleic acid, to allow for induction of *farE* in USA300, and the cells were then challenged with a 50 μM dose of linoleic acid, followed by monitoring of OD_{600}. After 30 minutes, each of USA300, USA300*farE::*ΦNE + pLI50, and USA300*farE::*ΦNE+ pLI*farE* exhibited evidence of continued growth (Fig. 2.10A). However, beyond 30 minutes, growth of USA300*farE::*ΦNE+ pLI50 was severely impaired, whereas USA300 continued to grow, and USA300*farE::*ΦNE+ pLI*farE* exhibited superior recovery. These data confirm that *farE* contributes to the ability of *S. aureus* USA300 to recover from an abrupt increase in the concentration of exogenous linoleic acid, and that conditions of the assay were not bactericidal.

We next wished to address the question of whether FarE was responsible for actively extruding linoleic acid from the *S. aureus* cell. To do this, we performed uptake assays using ^{14}C-linoleic acid. We performed these assays on cells that were treated the same as for the growth experiments described in Figure 2.10A, and cultures were supplemented with ^{14}C-linoleic acid, 30 minutes after challenge with 50 μM linoleic acid. Strikingly, USA300*farE::*ΦNE complemented with pLI*farE* exhibited the least accumulation of ^{14}C-linoleic acid, while USA300*farE::*ΦNE harboring the empty pLI50 vector exhibited the greatest accumulation, and wild type USA300 exhibited intermediate accumulation (Fig. 2.10B). Importantly, this reflected an inverse correlation between recovery of growth after exposure to 50 μM linoleic acid, and accumulation of ^{14}C-linoleic acid. Specifically, USA300*farE::*ΦNE + pLI*farE* exhibited the least accumulation of ^{14}C-linoleic acid, and

its growth was not adversely affected, whereas USA300*farE::*ΦNE + pLI50 exhibited the greatest accumulation and its growth was severely impaired, while wild type USA300 exhibited intermediate growth and accumulation kinetics. These data support the contention that FarE mediated efflux of unsaturated free fatty acids is required to support growth of *S. aureus* at elevated concentrations of antimicrobial fatty acid.

A. Growth Assay

Add 50 μM LA

Add ^{14}C-LA

OD$_{600}$

1

0.1

0   1   2   3   4

Time (h)

○ USA300
□ USA300*farE::*ΦNE + pLI50
■ USA300*farE::*ΦNE+ pLI-*farE*

B. Uptake Assay

^{14}C-Linoleic Acid pmol/ug

4

3

2

1

0

0   2   4   6   8   10

Time (m)

○ USA300
□ USA300*farE::*ΦNE + pLI50
■ USA300*farE::*ΦNE+ pLI-*farE*
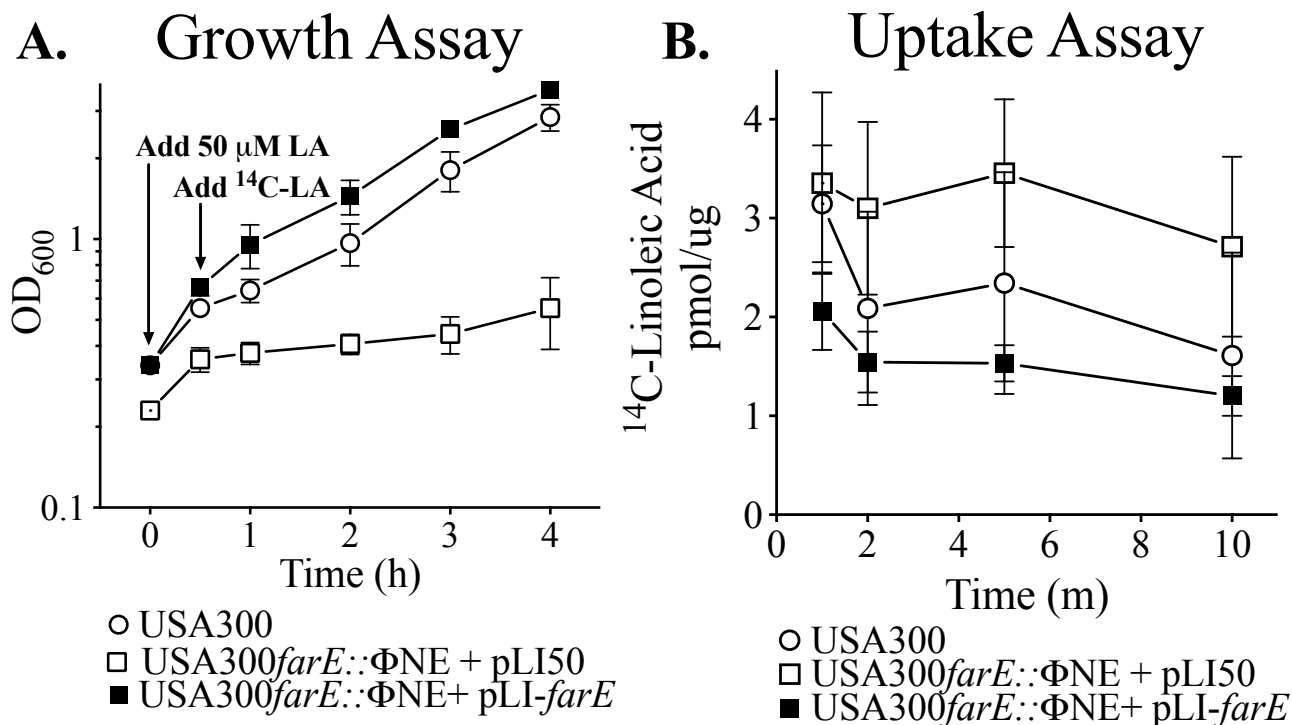
Figure 2.10 Growth (A) and uptake of ^{14}C-linoleic acid (B) following exposure of *S. aureus* USA300 and USA300*farE::*ΦNE to an increase in concentration of linoleic acid. A. quadruplicate cultures of USA300, USA300*farE::*ΦNE + pLI50, or USA300*farE::*ΦNE + pLI*farE* were grown in TSB + 20 μM linoleic acid to an OD$_{600}$ of approximately 0.2 to 0.3. The cultures were then supplemented with an additional 50 μM dose of linoleic acid, and growth (OD$_{600}$) was measured after 30 minutes, and then at hourly intervals. When this experiment was conducted for the purpose of quantifying uptake of ^{14}C-linoleic acid **(B)**, the cultures were supplemented with 0.20 μCi/mL of ^{14}C-linoleic acid at t = 30 minutes, and aliquots of culture were processed for quantification of ^{14}C-linoleic acid uptake at intervals of 1, 2, 5, and 10 minutes. Each data point represents the mean and standard deviation of quadruplicate samples.

2.4 Discussion

Through comparative genome sequencing of *S. aureus* USA300 variants that were selected for enhanced resistance to linoleic acid, we identified a regulator of fatty acid resistance, *farR*, and an effector of fatty acid resistance *farE*, which to our knowledge, is the first description of a dedicated and inducible mechanism of *S. aureus* resistance to antimicrobial fatty acids. These genes bear a similarity to the *acrR* and *acrB* paradigm in *E. coli*, where *acrR* and *acrB* were discovered through *in vitro* selection of acriflavine-resistant mutants, which mapped to the *acr* locus (40, 56, 57). The emergence of antibiotic resistance in Gram-negative bacteria has also been attributed to the *in vivo* selection of mutations in the transcriptional repressor *acrR*, which promote increased expression of the efflux pump encoded by *acrB* (58-60). Similarly, we discovered *farR* through *in vitro* selection of USA300 variants with increased resistance to linoleic acid. As with many proteins that possess an N-terminal TetR DNA binding domain, protein structural modeling and homology searches indicate that FarR belongs to the TetR/AcrR family of regulators, while FarE belongs to the RND-family of multi-drug efflux pumps, which include AcrB.

In addition to our own work which support a role for FarE as an efflux pump, other researchers using a different approach with *S. aureus* COL, demonstrated that an amino acid substitution in FarE (SACOL2566) promotes resistance to a newly described oxadiazole family of antibiotics (61). In *E. coli*, polymorphisms that cause amino acid substitutions in AcrB can also accrue during *in vitro* selection of strains that are resistant to fluoroquinolone antibiotics (62), and in these examples, it is likely that resistance is due to amino acid substitutions that expand the substrate specificity of the efflux pump (61, 62). However, although AcrB family efflux pumps have been most extensively characterized as mediators of multiple drug resistance, we contend that the primary function of FarE is to promote efflux of antimicrobial fatty acids that would be encountered during colonization, or within a tissue abscess. This is consistent with the belief that members of the AcrB family, which are encoded by the core genome, evolved to promote efflux of host-derived toxic compounds, including bile salts and fatty acids (63-68).

It is especially significant that expression of *farE* was most strongly induced by linoleic and arachidonic acid. Since *S. aureus* cannot synthesize unsaturated fatty acids (69), our data suggest that *farE* is induced as part of a signaling pathway that is activated by host-specific unsaturated free fatty acids. In other work, bactericidal assays conducted with human nasal secretions established that cholesterol esters of linoleic- and arachidonic acid were the principal bactericidal components towards *Pseudomonas aeruginosa*, which does not colonize the nose, but did not affect viability of *S. aureus* (16), and linoleic acid is also the principal antimicrobial fatty acid in homogenates of murine tissue abscesses (18, 51). Although arachidonic acid was not identified as a major fatty acid in abscess homogenates, it is a major unsaturated fatty acid in erythrocyte and leukocyte membrane phospholipid (23, 70), from which it is released by phospholipases at sites of infection, and rapidly converted to inflammatory mediators (71). Therefore, the induction of *farE* in response to linoleic- and arachidonic acid may represent an evolutionary feature that contributes to the success of *S. aureus* as a human pathogen.

Our observations are consistent with a requirement for FarE in maintaining membrane homeostasis when *S. aureus* is exposed to host-derived antimicrobial unsaturated free fatty acids. Since *S. aureus* cannot degrade exogenous fatty acids through β-oxidation, its primary means of coping with exogenous fatty acids is through incorporation into membrane phospholipid, which involves a novel fatty acid kinase pathway, whereby phosphorylated fatty acid is directly incorporated into glycerol-3-phosphate (54, 55). This in itself may represent a primary means of detoxifying long chain unsaturated free fatty acids, which promote loss of membrane integrity and cell death if allowed to accumulate in the cytoplasmic membrane (19). Importantly, *S. aureus* cannot synthesize unsaturated fatty acids, and maintains membrane fluidity through synthesis of branched chain fatty acids; primarily anteiso-C15 (69). From these considerations, we can envision two scenarios whereby FarE would be required under such conditions.

First, although some bacteria cease the *de novo* synthesis of fatty acids when provided with an exogenous supply of unsaturated fatty acids, this does not occur in *S. aureus*, which continues to synthesize fatty acids (72). However, under such conditions, there is reduced incorporation of endogenously synthesized anteiso-C15 into phospholipid, likely

due to displacement or competition from the exogenous unsaturated fatty acid (72, 73). Consequently, it is likely that unutilized metabolites will accumulate, which could be dealt with through an efflux mechanism, and at least one study has proposed that the primary function of an RND family efflux pump is to promote efflux of fatty acids that are replaced as a result of membrane damage or phospholipid turnover (74). Second, although incorporation of unsaturated fatty acids into phospholipid may comprise an effective means of detoxification, it would also promote an increase in membrane fluidity which if too severe, would compromise membrane function. In this context, we note from our analysis of uptake of ^{14}C-linoleic acid, that USA300*farE::*ΦNE cells exhibited significantly greater uptake of ^{14}C-linoleic acid compared to wild type USA300 (Fig. 2.10B). Therefore, although growth of USA300*farE::*ΦNE cells was impaired under these conditions (Fig. 2.10A), it continued to accumulate ^{14}C-linoleic acid, which suggests that there is sufficient metabolic capacity to incorporate unsaturated fatty acid into phospholipid, at a level that is beyond the tolerance for proper membrane function. Consequently, FarE function could also be required under such conditions, to ensure that incorporation of unsaturated fatty acid into phospholipid does not exceed a level of tolerance for membrane fluidity.

Although our data supported a role for *farE* in mediating resistance to linoleic and arachidonic acid, it did not confer resistance to palmitoleic acid, which is consistent with their being distinct mechanisms for resistance to unsaturated fatty acids of 16- and 18-carbon chain length. First, *S. aureus* exhibits a differential capacity to incorporate exogenous unsaturated 16- or 18-carbon fatty acids into membrane phospholipid. Oleic acid (C18:1) is directly incorporated into phospholipid (72), but palmitoleic acid must first be extended by the *S. aureus* fatty acid biosynthesis machinery, in a rate limiting step, to produce C18:1, which is then incorporated into phospholipid (19). Perhaps due to the less efficient incorporation of C16:1 fatty acids into phospholipid, *S. aureus* has evolved some capacity to exclude entry of palmitoleic and sapienic acid into the cytoplasm, due to cell surface teichoic acids and the low iron-induced cell surface protein IsdA, which function as a filtering mechanism to restrict penetration through the cell wall (19, 20). Others also reported that a major facilitator superfamily efflux pump encoded by *tet38* promoted resistance to palmitoleic acid (21), and although we were not able to

confirm this through construction of a USA300Δtet38 deletion mutant, it may be that *tet38* functions in a strain specific context.

Further relevant to these considerations, expression of *tet38* was induced primarily by palmitoleic acid, and much less effectively by linoleic acid, whereas we observed the opposite response for induction of *farE*. Importantly, with our identification of a SNP in *farR* that promotes increased expression of *farE*, we have provided the first mechanistic description of an efflux pump that is specifically induced in response to antimicrobial fatty acids in *S. aureus*, and at a broader level, in Gram-positive bacteria. FarR belongs to the TetR/AcrR family of transcriptional regulators, which usually repress transcription of divergent genes, by virtue of an N-terminal DNA binding domain that recognizes a specific operator site in the promoter segment of a target gene, and the affinity of this interaction is modulated by a C-terminal domain that binds a small inducing ligand (75, 76). In a relevant example, FadR of *Thermus thermophilus* represses expression of genes required to degrade fatty acids, which are de-repressed upon binding of an acyl-CoA ligand to FadR (46). However, although *farE* is induced by antimicrobial fatty acids, we cannot yet conclude that *farR* is alone sufficient to regulate *farE*. If FarR functioned strictly as a repressor, then inactivation of *farR* should have caused de-repression of *farE*. However, this was not observed, and *farR* was in fact needed for induction of *farE* (Fig. 2.1). Conversely, FAR7 exhibited a constitutive measure of *farE* expression, attributed to the $H_{121}Y$ substitution in FarR, which also conferred a significantly higher induced level of *farE* expression than could be achieved in wild type USA300 (Fig. 2.8B).

As this substitution is not within the N-terminal DNA binding domain, which spans amino acids 28-61 of FarR, it should not directly affect the DNA binding function. However, in a potentially related example, FadR represses expression of genes required for β-oxidation of fatty acids, and the conformation of amino acids 106-119 in the C-terminal domain underwent a significant shift on binding of fatty acid, including R^{109}, which had an important role in maintaining the DNA-binding affinity, even though it is not within the N-terminal DNA binding domain (77). Therefore, the $H_{121}Y$ substitution in FarR could still affect the function of the N-terminal DNA binding domain, or alternately

it may affect the ability of FarR to form functional oligomers; typically dimers or tetramers, which is another characteristic trait of the TetR family of regulators (75, 78).

Although most TetR regulators repress expression of divergently transcribed genes (75, 78), our observation that FarR is required for induction of *farE* is not unprecedented, and FarR may resemble a limited number of TetR regulators that trigger a broader cellular response to environmental insults (78-83). In one such example, the SczA metal ion-dependent transcriptional regulator of *Streptococcus pneumoniae* (82) binds to a specific operator site to repress transcription of a target gene in the absence of zinc, but when zinc is present, it binds to a different DNA segment upstream of the regulated gene to activate transcription. Alternatively, FarR may still function as repressor of *farE* in the absence of inducer, and then in the presence of exogenous fatty acid may serve to promote expression of a positive acting transcription factor that is needed to activate *farE*. This would partially conform to the AcrR-AcrB paradigm, where AcrR ensures that *acrB* is not expressed in the absence of an inducing stimulus, but other positive acting factors are required to activate *acrB* (84-86). With these considerations in mind, work is in progress to determine the mechanism of FarR-dependent regulation of gene expression, through analysis of its interaction with different fatty acids and target promoters, and the scope of genes that are affected by this interaction.

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Chapter 3

3 Identification of FarR binding sites responsible for auto-repression and efflux-mediated resistance to antimicrobial fatty acids in *Staphylococcus aureus*[2]

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[2] This manuscript is in preparation.

3.1 Introduction

Methicillin-resistant *Staphylococcus aureus* has rapidly achieved pandemic status in community and hospital settings. To persist on human hosts, *S. aureus* must have intrinsic defense mechanisms to cope with antimicrobial unsaturated free fatty acids (uFFA), an important component of human innate defense mechanisms. In previous work, we identified a regulator of fatty acid resistance, FarR, which belongs to the TetR family of transcriptional regulators (TFR) (1). TFRs represent the third most ubiquitous transcriptional regulators in prokaryotes (2). This family is named after the prototypic TetR that has a role in mediating tetracycline resistance (3). Members of this family typically exert their effect on divergent genes in response to small hydrophobic ligands and are involved in the transcriptional regulation of a wide range of biological functions such as multidrug resistance, antibiotics biosynthesis, quorum sensing, and pathogenicity (4). Studies show that overexpression of multidrug efflux pumps, and the resulting antibiotic resistance, is often attributed to mutations in transcriptional regulators such as those belonging to the TetR family. In Gram-negative bacteria, AcrAB is an RND-efflux pump regulated by the TFR, AcrR, and other global transcriptional regulators such as MarA, SdiA, RobA and SoxS (5, 6). In this paradigm, the de-repression of AcrR in the presence of an inducing ligand is not enough to ensure that the pump is only expressed when needed, and additional positive-acting factors are needed to fully activate the efflux pump (7). As a result the TFR AcrR fine-tunes the expression of the efflux pump, as opposed to straightforward repression and de-repression (8). Nevertheless, mutations in *acrR* alone can cause overexpression of the AcrAB efflux pump and are linked to antibiotic resistance in clinical isolates (9). Similar to the *acrR* and *acrB* paradigm in *E. coli*, where *acrR* and *acrB* were discovered by *in vitro* selection of acriflavine-resistant mutants, we discovered *farR* through *in vitro* selection of USA300 variants with increased resistance to linoleic acid (1).

Analysis of the region upstream of *farR* revealed an RND efflux pump, *farE*, the expression of which is induced by antimicrobial uFFA, including linoleic and arachidonic acid. Importantly, a single nucleotide polymorphism causing a $His_{121}Tyr$ substitution in FarR caused increased expression of *farE*, in the absence of an inducing stimulus, and a

significantly higher induced level of expression when cells are exposed to linoleic acid. Moreover, we showed that *farE* is required to support the growth of *S. aureus* in the presence of sub-inhibitory concentrations of linoleic acid, and that resistance to linoleic acid is proportional to *farE* expression (1).

The regulation of lipid homeostasis is essential to maintaining bacterial physiology and here we report the first steps towards understanding how FarR regulates the expression of *farE*. We previously established that *farE* is induced by antimicrobial fatty acids in a *farR*-dependent manner, providing evidence that *farR* does not function strictly as a repressor. TFRs are mainly repressors in nature, with limited reports of unconventional TFRs that have dual roles (10–12). SczA of *Streptococcus pneumoniae*, for instance, is an unconventional TFR that binds an operator site in the absence of its ligand, zinc, and binds another distinct operator site further upstream to act as an activator and help recruit the transcription machinery (13). We here examine FarR-DNA interaction and establish that FarR binds to distinct operator sites in the intergenic segment between the divergent *farE* and *farR*, and nucleotide mutation in these operator sites abrogated protein binding of FarR to these sites. Using a gene-reporter system, we determine that FarR, similar to the majority of TFRs, is auto-regulatory in nature and that the operator site responsible for auto-repression spans the -10 element of P_{farR} as well as +1 transcriptional start site of *farR*. Finally, we establish that FarR binds to a DNA operator site upstream of the putative *farE* promoter that may have a role in *farE* activation.

3.2 Materials and methods

3.2.1 Bacterial strains and growth conditions

A list of bacterial strains used in this study is provided in Table 3.1. Bacterial cultures were maintained as frozen stocks at -80°C in 20% glycerol and streaked on tryptic soy agar (TSA) plates (*S. aureus*) or Luria Bertani (LB) agar plates (*E. coli*), when needed. Antibiotics were added when needed at the following concentrations: chloramphenicol, 5 μg/ml; erythromycin, 5μg/mL; anhydrotetracycline, 250 ng/ml; ampicillin, 100 μg/mL and kanamycin, 50 μg/mL. For growth analyses, single colonies were inoculated into 3 mL TSB or LB broth supplemented with antibiotics, when required, and incubated

overnight at 37°C on an orbital shaker (VWR). Cultures were then diluted into 25 mL TSB to a starting OD_{600} of 0.01 and incubated at 37°C on an orbital shaker at 180 rpm. For fatty acids supplementation, 25 mL TSB supplemented with 0.1% DMSO was used.

3.2.2   Construction of *S. aureus* USA300Δ*farER* and USA300Δ*fakA*

In-frame, markerless deletion of both *farE* and *farR* as well as *fakA* was achieved using pKOR1 as previously described (14). Briefly, 1000 bp segments located upstream and downstream of the *farE-farR* locus were amplified using PCR primers that incorporate *attB1* and *attB2* sequences as well as a *Sac*II cut site to allow for ligation of the resulting amplicons. The segment upstream of *farE* was amplified using *farE*-UP-*attB1* that incorporates *attB1* sequence on the 5′-end of the segment, and *farE*-UP-*Sac*II that incorporates a *Sac*II cut site on the 3′-end of the segment. The segment downstream of *farR* was amplified using *farR*-DW-*Sac*II that incorporates a *Sac*II site at the 5′-end of the segment, and *farR*-DW-*attB2*, which incorporates an *attB2* sequence on the 3′-end of the segment. Similarly, the *fakA* mutant was constructed by amplifying 1000 bp segments located upstream and downstream of *fakA* using PCR primers that incorporate *attB1* and *attB2* sequences as well as a *Sac*II cut site to allow for ligation of the resulting amplicons. The amplicons were subsequently purified, digested with *Sac*II and ligated together using T4 DNA ligase (NEB). The resulting fragment was then introduced into the pKOR1 vector using site-specific recombination. This was achieved using BP Clonase II (Invitrogen) to facilitate recombination between the *attB1* and *attB2* sequences on the ligated PCR product and the *attP1* and *attP2* sites on the vector, generating pKORΔ*farER* or pKORΔ*fakA*. The vectors were constructed in *E. coli* DH5α background first and subsequently passaged through *S. aureus* RN4220 at 30°C before electroporation into *S. aureus* USA300. In-frame allelic replacement of *farER* or *fakA* was then achieved by a two-step temperature shift and anti-sense counter-selection as previously described (14). The USA300Δ*fakA*-Δ*farER* double mutant was constructed by electroporating pKORΔ*farER* into *S. aureus* USA300Δ*fakA*, followed by in-frame allelic replacement as described above.

3.2.3 Construction of complementation and reporter gene constructs

Oligonucleotides used to generate reporter gene constructs, or to complement mutants can be found in Table 3.2. For complementation, we utilized the pLI*farR* and pLI*farE* complementation vectors that we previously made (1). *farE* was cut out of pLI*farE* using the restriction enzymes *Kpn*I and *Sac*II, and subsequently gel purified for ligation with pLI*farR* in *E. coli* DH5α background. The presence of ligated genes (*farER*) was confirmed by PCR and DNA sequence analysis. The pLI*farER* construct was then passaged through *S. aureus* RN4220 and electroporated into USA300Δ*farER*. The complementation construct pLI*farR* was modified using the mutagenic primers P$_{farR}$-10G>A-F and P$_{farR}$-10G>A-R, using protocols and reagents following the QuikChange Site-Directed Mutagenesis Kit (Stratagene), to construct pLI*farR*$_{TAG>A}$, harboring a single nucleotide substitution within the -10 motif of the P$_{farR}$ promoter. To construct reporter gene constructs we utilized the pGY*lux* vector where the luciferase operon can be driven from our promoter of interest (15). We previously constructed *farE::lux* by cloning a 397 bp fragment, which included the entire intergenic segment between *farE* and *farR* and additional sequence from the 5′-end of *farE* and 5′-end of *farR*, in front of the *lux* operon (1). To localize the *farR* promoter, we cloned two *farR* segments into the pGY*lux* vector; the larger segment (246 bp) that made up pGY*farR::lux1* was amplified using primers pGY*farR1*-F and pGY*farR*-R, and the smaller segment (164 bp) that made up pGY*farR::lux*2 was amplified using primers pGY*farR2*-F and pGY*farR*-R. Both segments were cloned into the *BamH*I and *Sal*I sites of pGY*lux* (15). pLI*farR*$_{TAG>A}$ was used as a template in PCR with primers pGY*farR1*-F and pGY*farR*-R, and the amplicon was then cloned in pGY*lux* to generate pGY*farR*$_{TAG>A}$*::lux*.

Table 3.1 Strains and plasmids used in Chapter 3.

Strain or plasmid	Description	Source
<u>Strains:</u>		
S. aureus:		
USA300LAC	Community-acquired methicillin-resistant *S. aureus*, wild-type strain cured of antibiotic resistance plasmids	(16)
RN4220	Restriction endonuclease deficient lab strain of *S. aureus* capable of accepting foreign DNA	(17)
USA300Δ*fakA*	USA300LAC with markerless deletion of *fakA* (SAUSA300_1119)	This study
USA300Δ*farER*	USA300LAC with markerless deletion of *farE* (SAUSA300_2489) and *farR* (SAUSA300_2490)	This study
USA300Δ*fakA*-Δ*farER*	USA300LAC with markerless deletion of *fakA, farE,* and *farR*	This study
USA300Δ*farER* (pLI*50)*	USA300Δ*farER* with empty pLI50 vector, Cm^r	This study
USA300Δ*farER* (pLI*farE)*	USA300Δ*farER* complemented with native *farE,* cloned in pLI50, Cm^r	This study
USA300Δ*farER* (pLI*farR)*	USA300Δ*farER* complemented with native *farR,* cloned in pLI50, Cm^r	This study
USA300Δ*farER* (pLI*farR7)*	USA300Δ*farER* complemented with *farR* from SNP-containing clone FAR7, cloned in pLI50, Cm^r	This study
USA300Δ*farER* (pLI*farER)*	USA300Δ*farER* complemented with native *farER,* cloned in pLI50, Cm^r	This study
E. coli:		
DH5α	λ⁻ φ80d*lacZ*ΔM15Δ(*lacZYA-argF)U169 recA1 endA1 hsdr17*(r$_K^-$ m$_K^-$) *supE44 thi-1 gyrA relA1*	Invitrogen
M15[pREP]	F-, Φ80ΔlacM15, thi, lac-, mtl-, recA+, KmR	Qiagen
<u>Plasmids:</u>		
pLI50	*E. coli-S. aureus* shuttle vector	(18)
pLI*farE*	pLI50 with native *farE* gene	(1)
pLI*farR*	pLI50 with native *farR* gene	(1)
pLI*farR7*	pLI50 with *farR* gene from the variant FAR7 clone	(1)
pLI*farR*$_{TAG>A}$	pLI50 with *farR* gene harboring a TAG>A mutation in the -10 element of P$_{farR}$	This study

pKOR1	*E. coli-S. aureus* shuttle vector containing $P_{xyl-tetO}$ and expressing antisense *secY* RNA	(14)
pKORΔ*farER*	pKOR-1 containing upstream and downstream flanking sequences for deletion of *farER*	This study
pKORΔ*fakA*	pKOR-1 containing upstream and downstream flanking sequences for deletion of *fakA*	This study
pGY*lux*	*E. coli-S. aureus* shuttle vector harboring promoterless *luxABCDE* operon	(15)
pGY*farE::lux*	*E. coli-S. aureus* shuttle vector containing P_{farE} for *luxABCDE* operon	(1)
pGY*farR::lux*1	*E. coli-S. aureus* shuttle vector containing P1 of *farR* for *luxABCDE* operon	This study
pGY*farR::lux*2	*E. coli-S. aureus* shuttle vector containing P2 of *farR* for *luxABCDE* operon	This study
pGY*farR*$_{TAG>A}$*::lux*	*E. coli-S. aureus* shuttle vector containing mutated P1 of *farR* for *luxABCDE* operon	This study
pQE30	*E. coli* vector for IPTG-inducible expression and incorporation of an N-terminal 6×His tag	Qiagen
pQE6His-*farR*	pQE30 containing 6×His tagged *farR*	This study
pQE6His-*farR7*	pQE30 containing 6×His tagged *farR* from the variant FAR7 clone	This study
pUC18	*E. coli* cloning vector	(19)

Table 3.2 Oligonucleotides used in Chapter 3.

Oligonucleotide	Sequence 5′ to 3′
farR-DW-*Sac*II[a]	ggacct**ccgcgg**GGCGAAGATATTGATAACATTTTCC
farR-DW-*attB2*	ggggaccactttgtacaagaaagctgggtGGTAAATTAGAACAAGGTGGCG
farE-UP-*attB1*	ggggacaagtttgtacaaaaaagcaggctTTCCTTTGCCTGTACGTGC
farE-UP-*Sac*II[a]	ggacct**ccgcgg**AACGATGGCATTGTACCAAG
fakA-UP-*attB1*	ggggacaagtttgtacaaaaaagcaggctGCGTGTGAACGTCTGTTACCAGTCGAAGC
fakA-UP-*Sac*II[a]	ggacct**ccgcgg**CATTTCAAGTTGTCCTCCTAAGCTTTCTTGC
fakA-DW-*Sac*II[a]	ggacct**ccgcgg**GTTCATGAAGGTGGACAACCAATTTATC
fakA-DW-*attB2*	ggggaccactttgtacaagaaagctgggtGATGACTTTTCTAATCTATTTAGCCATTGC
pGY*farE*-F[b]	ccc**ggatcc**TTGTACGGTGTACGAGTGCG
pGY*farE*-R[c]	ccc**gtcgac**CGGTGCATTTGTAGCAAGTG
pGY*farR1*-F[b]	ccc**ggatcc**TGCAGCTACAATCACTATCCATGC
pGY*farR2*-F[b]	ccc**ggatcc**GCCAAAGTATATTGCCTCC
pGY*farR*-R[c]	ccc**gtcgac**TAAATCAGTCTCTTTCATCTACATTTCTCC
farR-GSP1	TTATCTGGGATGTCGCTG
farR-GSP2	CCCGTCGACTCAGCGTCTTCTTCTTGG
farR-GSP3	ATTGTCGACACTCATCGTTGGAATGG
AAP	GGCCACGCGTCGACTAGTACGGG/ideoxyI/ideoxyI/GGG/ideoxyI/ideoxyI/GGG/ideoxyI/ideoxyI/G
AUAP	GGCCACGCGTCGACTAGTAC
M13-F	CGCCAGGGTTTTCCCAGTCACGAC
M13-R	AGCGGATAACAATTTCACACAGG
farR-6H-F[d]	CTACACACAAAGGAGAAATGTA**gagctc**ATGAAAGAGACTGATTTACGAG
farR-6H-R[e]	GGTAACGCTCATGAGTTTCT**aagctt**CTATTTAATCTTAATATTGATTAATCTATGG
OP$_E$-F	GTTTTTCAATCTTTTTATTCGTATCTAACG
OP$_E$-R	GATGGGGACATTCATCGC
OP$_{IN}$-F	CCCCATCTTATATAAAAATTTTGCC
OP$_{IN}$-R	CTACATTTCTCCTTTGTGTGTAG
OP$_R$-F	GATGAAAGAGACTGATTTACGAG
OP$_R$-R	CATATTTATCATAAAAATGTTTATAAATGTTGTACGG
OP1	GTTATAGTTTAAATATACAGTGTAGATTATTGTTCGATTATAG
OP2	GTATATTGCCTCCTTTTAAAATCAACGTTATAGTTTAAATATA
OP3	TTATTGTTCGATTATAGTATCTATCCCCGACCTCTTAAAGAAT
OP4	GAATCAATTGGAAAATTTTGTATATTAAACTACACACAAAGGAGAAATGTAG
OP5	AGTTTAAATATACAGTGTAGATTATTGTT
OP5A	AGTTTAAATATACA**ACACCA**ATTATTGTTCG

119

OP5TAG>A	AGTTTAAATATACAGTGTAAATTATTGTT
OP5ΔTAG	AGTTTAAATATACAGTGATTATTGTT
OP6	AAATCAACGTTATAGTTTAAATATACAG
OP7	TGTAGATTATTGTTCGATTATAGTATCT
OP11	GTATATTGCCTCCTTTTAAAATCAACGTTA
OP12	GCCTCCTTTTAAAATCAACGTTATAGTTTA
OP13	TTTTAAAATC**AACGTT**ATAGTTTAAATATA
OP13A	TAAAATC**CATTGT**ATAGTTTAAAT
OP13TAG>A	TTTTAAAATCAACGTTATAATTTAAATATA
P*farR*-10G>A-F	CCTTTTAAAATCAACGTTATAATTTAAATATACAGTGTAG
P*farR*-10G>A-R	CTACACTGTATATTTAAATTATAACGTTGATTTTAAAAGG

Lower case nucleotides are *attB2* and *attB1* site for cloning into pKOR1 vector. Lower case and bold nucleotides indicate the addition of 5' sequences to incorporate restriction endonuclease cut sites as follows [a]*Sac*II, [b]*BamH*I, [c]*Sal*I, [d]*Sac*I, and [e]*Hind*III. Nucleotides in bold show the locations of site-directed mutations.

3.2.4   RNA isolation and 5′-rapid amplification of complementary DNA ends

RNA was isolated from *S. aureus* cells (USA300 and FAR7) grown to mid-exponential phase (OD_{600} of 0.5) in TSB supplemented with 20 μM linoleic acid, using Aurum™ total RNA kit (Bio-Rad) as per manufacturer's instructions. Synthesis of first-strand cDNA was prepared using 1 μM *farR*-gene specific primer *farR*-GSP1, 1.5 μg of RNA, 50 μM dNTPs, and 20 units of SuperScript™ II reverse transcriptase (Invitrogen), followed by incubation at 42°C for 50 minutes. The original mRNA template was subsequently removed by treatment with a mixture of RNaseH (0.5 units) and RNase T1 (50 units) at 37°C for 30 minutes. The cDNA was then purified using BioArray® cDNA purification kit (Enzo Life Sciences) as per manufacturer's instructions to eliminate unincorporated *farR*-GSP1 and dNTPs. A dC-tail was added to the 3′-end of the purified cDNA using 20 units of terminal transferase (Roche) and 0.5 mM CTP, and the resulting C-tailed cDNA was utilized in a PCR reaction using abridged anchor primer (AAP) and nested *farR* gene specific primer *farR*-GSP2. A second PCR was then conducted, using the first PCR product as a template, with abridged universal amplification primer (AUAP) and a nested *farR*-GSP3 containing a *Sal*I restriction cut site to allow for cloning into pUC18. Cloned products were sequenced using M13-F and M13-R primers that flank the multiple cloning site of pUC18. The +1 transcription start site was identified as the first nucleotide following the poly C- or G-tail, depending on the orientation of the cloned insert.

3.2.5   Construction and purification of recombinant FarR

farR genes from both USA300 and FAR7 were cloned in pQE30 to allow for expression with an N-terminal 6×His-tag using primers *farR-6H*-F and *farR-6H*-R. Recombinant proteins were expressed and purified from *E. coli* M15[pREP]. Bacterial cultures were grown at 37°C in LB (Sigma Aldrich) supplemented with 100 μg/mL ampicillin and 50 μg/mL kanamycin, to an OD_{600} of 0.8 before the addition of 0.1 mM of isopropyl 1-thio-β-D-galactopyranoside (IPTG) and incubation at room temperature with shaking for additional 18 hours. Cells were harvested by centrifugation and resuspended in binding

buffer (20 mM sodium phosphate, 0.5 M NaCl, 40 mM imidazole, pH 7.4), lysed in a cell disruptor (Constans System Ltd.) at 25000 psi, and subsequently pelleted at 3000 rpm for 15 minutes. Supernatant was then ultracentrifuged for 50 minutes at 50,000 xg in a Beckman Coulter Optima L-900K ultracentrifuge, after which the soluble fraction was filtered through a 0.45µm Acrodisk® syringe filter (Pall Laboratory). The lysate was applied onto a 1 mL His-Trap nickel affinity column (GE Healthcare) that was equilibrated with binding buffer. After washing extensively with binding buffer, bound His-tagged protein was eluted over a linear imidazole gradient up to 0.5 M imidazole in 20 mM sodium phosphate. Column fractions were assessed by SDS-polyacrylamide gel electrophoresis to check for purity, and fractions were then pooled and dialyzed in 20 mM sodium phosphate, 0.5 M NaCl, pH 7.4 at 4°C overnight (Appendix 8). Protein concentration was determined by Bradford assay using Bio-Rad protein assay reagent.

3.2.6   FarR-DNA interaction studies

Electrophoretic mobility shift assays (EMSA) were performed using recombinant 6×His-tagged FarR and fluorescently-labeled duplex oligonucleotides probes. A list of oligonucleotides used in this study is provided in Table 3.2. IRDye-labeled single-stranded oligonucleotides were purchased from Integrated DNA technologies (IDT®) and complementary oligonucleotides were annealed at 100 µM each in 10 mM Tris, pH 8.0, 0.1 mM EDTA, by incubation at 95°C for 5 minutes followed by cooling down slowly to room temperature for 45 minutes. Each 25 µL EMSA reaction contained 5 pmol of fluorescently-labeled probe, up to 2 µM purified FarR, 240 µg/mL bovine serum albumin (BSA) and 15.2 µg/mL poly[d(I-C)] in 10% glycerol, 15 mM Tris-HCl pH 8.0, 0.5 mM $MnCl_2$, 60 mM KCl, 0.5 mM $MgCl_2$, and 8 mM dithiothreitol (DTT). Reaction mixtures were incubated at room temperature for 60 minutes, after which they were run on a 6% TBE-acrylamide gel for 45 minutes at 120V, and imaged using an Odyssey imager (LI-COR Biosciences). FarR-DNA interaction was determined as the conversion of unbound DNA probe to slow-moving protein-DNA complex. In competition assays, unlabelled competitor probes harboring specific nucleotide substitutions were added in 50-fold excess compared to that of the labelled probes.

3.2.7 Antibody production and western blotting

Rabbit polyclonal antisera recognizing FarR were generated by ProSci Incorporated (Poway, CA, USA). Two hundred μg of recombinant 6×His-FarR emulsified in complete Freund's Adjuvant was used for the initial injection of rabbits, and 100 μg of the recombinant protein emulsified in incomplete Freund's Adjuvant was subsequently used for each booster immunization. Rabbits were immunized every other week for a total of six weeks. For western blotting, cell lysates of *E. coli* complemented with pLI50 harboring native *farR* and *farR* bearing mutated P*farR* were prepared by incubating washed cells with lysis buffer (150 mM NaCl, 5 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0, 1% (v/v) Triton X-100, 0.5% (v/v) SDS), supplemented with EDTA-free protease inhibitor cocktail (Roche), at room temperature for two hours with agitation, followed by centrifugation at 4,200 x g for 20 minutes. After determining protein content of the clarified cell lysate, samples containing 25 μg of total cell lysate protein were subjected to SDS-PAGE using a 12% polyacrylamide resolving gel. Proteins were then transferred to PVDF membrane following standard protocols. Primary anti-FarR antiserum was used at a dilution of 1:5000 followed by secondary IRDye800-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories Inc.). Membranes were imaged using Odyssey imager (LI-COR Biosciences).

3.2.8 Murine infection model

We utilized a skin abscess infection model as described by Malachowa *et al.* with adaptation (20). Briefly, female BALB/c mice were anesthetized by an isoflurane vaporizer, and flanks were treated with Nair hair removal lotion first, then shaved by a battery-operated trimmer one day prior to the inoculation to allow for proper visualization and measurement of abscesses. On infection day, bacterial cultures were inoculated at an OD_{600} of 0.01 from an overnight stationary phase culture, and grown at 37°C with shaking to an OD of 2.0. Bacteria were harvested by centrifugation, washed twice with sterile PBS, and resuspended in sterile PBS to obtain a final concentration of $2x10^8$ cfu/mL. Bacterial suspensions were kept on ice until injection. Colony forming units in

each suspension were confirmed by plating on TSA and enumerating the next day. Mice were anesthetized by an isoflurane vaporizer, weighed, then challenged by subcutaneous injection of 50 μl of bacterial suspension (1×10^7 cfu), or PBS only. Abscess progression was pictured daily to quantify abscess area by Image J. Animal weight was also monitored daily for three days, after which the animals were sacrificed, and the lesions were imaged. The infected lesions were then excised, homogenized in PBS-0.01% Triton X-100, and plated on Mannitol Salt Agar (MSA) for enumeration.

3.2.9   Computer analyses

Protein structural modeling was done using PHYRE2 (21). Analysis of DNA sequences and primer design were done using MacVector (Version 14.0.4 MacVector Inc.). Promoter predictions were performed using the Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/promoter.html), with adjustments for detection of prokaryotic promoters. Multiple sequence alignment was done using ClustalW 1.4 (22). Data points for growth, viability, and luciferase reporter gene assays were plotted and analyzed using GraphPad Prism, version 7.0. Statistical significance was determined using unpaired one-tailed Student's *t*-test and two-way ANOVA using the statistical feature of GraphPad Prism, version 7.0.

3.3 Results

3.3.1   Deletion of *farER* results in loss of inducible resistance to linoleic acid

In our previous work, transposon insertions in *farE* and *farR* were used to demonstrate that the *farE* efflux pump, and the divergent transcriptional regulator *farR* were both required for inducible resistance of USA300 to the bactericidal activity of 100 μM linoleic acid (1) . To confirm these observations, and to enable more detailed studies on gene expression and structure-function relationships USA300Δ*farER*, where both genes are deleted, was constructed using pKOR1 markerless mutagenesis. In agreement with our previous observations, USA300Δ*farER* suffered a loss of resistance to the bactericidal activity of 100 μM linoleic acid (Fig. 3.1A).

3.3.2   *farR* is required for *farE* induction

The conventional mechanism of action of TFRs is to repress the expression of divergent genes. In previous work, we have shown that when assaying *farE::lux* reporter gene construct in USA300, the reporter activity was negligible in TSB only, but induced in TSB supplemented with 20 μM linoleic acid. We also observed that the reporter activity was negligible in USA300*farR*::ΦNE background suggesting that *farR* is required for induction of *farE* expression. Additionally, USA300Δ*farER* was unable to grow in TSB supplemented with 50 μM linoleic acid, and complementing with *farE* alone did not rescue growth, whereas complementing with pLI*farER* restored growth over 8 hours of incubation (Fig. 3.1B). As expected, when assaying *farE::lux* activity in USA300Δ*farER*, there was negligible induction of *farE* in the absence of *farR* function (Fig. 3.2). These data confirm that functional *farR* is indeed required for expression of *farE*.

A. Viability in 100 μM LA

B. Growth in 50 μM LA

● USA300
□ USA300Δ*farER*
■ USA300Δ*farER* + pLI*farER*

□ USA300Δ*farER* + pLI50
○ USA300Δ*farER* + pLI*farE*
■ USA300Δ*farER* + pLI*farER*
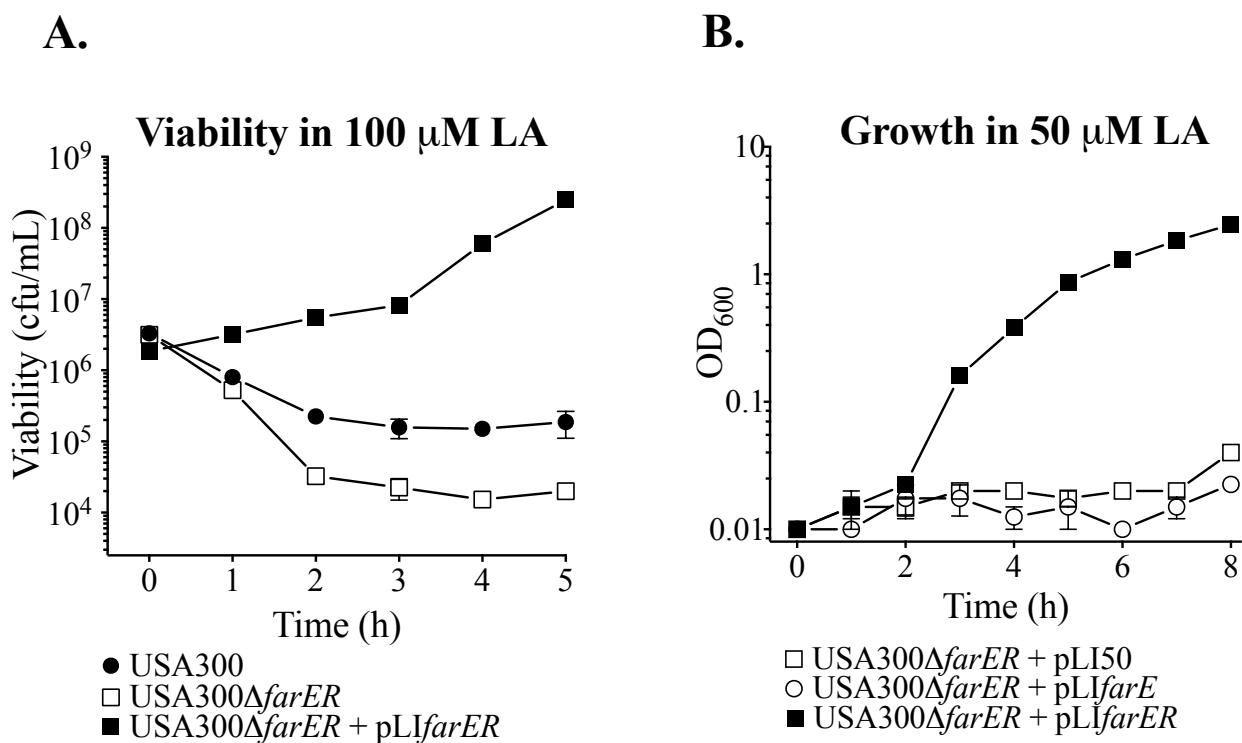
Figure 3.1 *farER* contributes to resistance of *S. aureus* USA300 to linoleic acid. A.
Bactericidal assay: USA300, USA300Δ*farER*, or USA300Δ*farER* + pLI*farER* were
grown to mid-exponential phase in TSB + 20 μM linoleic acid, and then diluted to 10^6
cfu/mL in fresh TSB containing 100 μM linoleic acid. Viable cell counts were taken at
hourly intervals. **B.** Growth assay: USA300Δ*farER* complemented with pLI*farE*,
pLI*farER*, or pLI50 vehicle was inoculated into TSB + 50 μM linoleic acid, and growth
(OD_{600}) was monitored at hourly intervals. Each data point represents mean value of
quadruplicate cultures. Error bars represent Standard Error of the Mean.
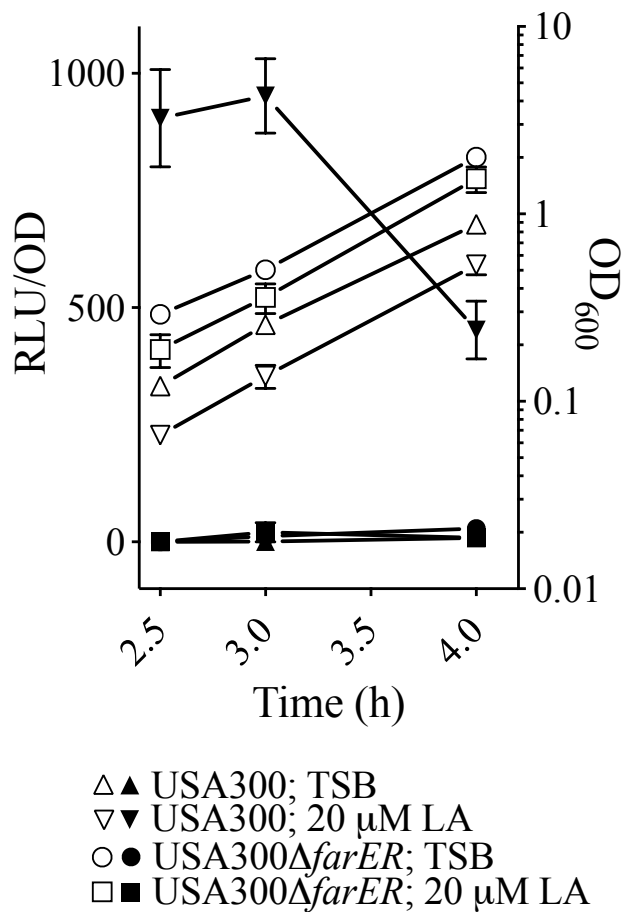
Figure 3.2 *farR* is required for *farE* induction. Growth (OD_{600}; open symbols) and relative luminescence units (RLU/OD; closed symbols) of USA300 and USA300Δ*farER* harboring the pGY*farE::lux* reporter construct are shown. Strains were grown in TSB or TSB supplemented with 20 μM linoleic acid (LA). Each value represents the mean and standard deviation of three separate cultures, and each culture was subjected to quadruplicate luminescence readings at each time point. Error bars represent Standard Error of the Mean.

3.3.3   Identification of *farR* promoter and transcription start site

The neighboring genes *farE* and *farR* are divergently transcribed. The intergenic segment between *farE* and *farR* translational start sites is 145 nucleotides, and bioinformatics analyses identified two potential *farR* promoters, P1 and P2, in this intergenic segment. P1 overlaps with the *farE* translational initiation codon on the minus strand, and is located 145 bp upstream of the translational initiation codon of *farR*, whereas P2 is located 132 bp upstream of the translational initiation of *farR* (Fig. 3.3A). To confirm P$_{farR}$ in the context of transcriptional initiation of *farR*, we used 5′-RACE to determine the +1 site of *farR*. The transcription start site of *farR* was located at 104 bp from the translation start site in two out of three independent experiments, and one experiment showed that the transcription start site was 105 bp from the gene's start codon. These results correspond to P2 being the primary P$_{farR}$ (Fig. 3.3A). To confirm this, we designed two overlapping P$_{farR}$ segments to clone reporter gene fusions in pGY*lux*. The larger construct, *farR::lux*1, harbors structural elements of both promoters P1 and P2 whereas the shorter truncated construct, *farR::lux*2, lacks the -35 element of P1. When these reporter constructs were assayed in *E. coli*, both displayed luminescence activity but the truncated *farR::lux*2 exhibited 8-fold less activity compared to that of the larger *farR::lux*1 construct (Fig. 3.3B). These data suggest that P2 is sufficient to promote transcription of the gene, although contribution of the overlapping P1 cannot be excluded. In a similar fashion, bioinformatics analyses identified a putative *farE* promoter in the *farER* intergenic segment that is located 102 bp upstream of the translational initiation codon of *farE*; however, 5′-RACE experiments failed to determine the +1 transcription start site of *farE*.

3.3.4   Expression of *farR* is subject to auto-regulation

Members of the TetR family of transcriptional regulators are typically subject to auto-regulation, including CamR in *Pseudomonas putida*, TcmR in *Streptomyces glaucescens* and the well-characterized TetR. Conversely, QacR of *S. aureus* was not subject to auto-regulation (23). To determine whether *farR* is auto-regulated, we assayed *farR::lux*1 activity in both USA300 and USA300Δ*farER* backgrounds, and observed that the reporter activity was negligible in USA300, but was strongly de-repressed in

USA300Δ*farER*. These data support *farR* auto-regulation such that *farR::lux* reporter activity is higher in the USA300Δ*farER* background, due to the absence of functional FarR, concomitant with de-repression of P*farR*. (Fig. 3.3C).

A.

P1 P2 *farR*

-35 -35 -10 -10 +1

RBS

farE

-10 -35

P*farE*

RBS

farE *farR*

B. *farR::lux* Activity in *E. coli*

RLU/OD

1.2×10^7

8.0×10^6

4.0×10^6

0.0

farR::lux1 *farR::lux2*

*
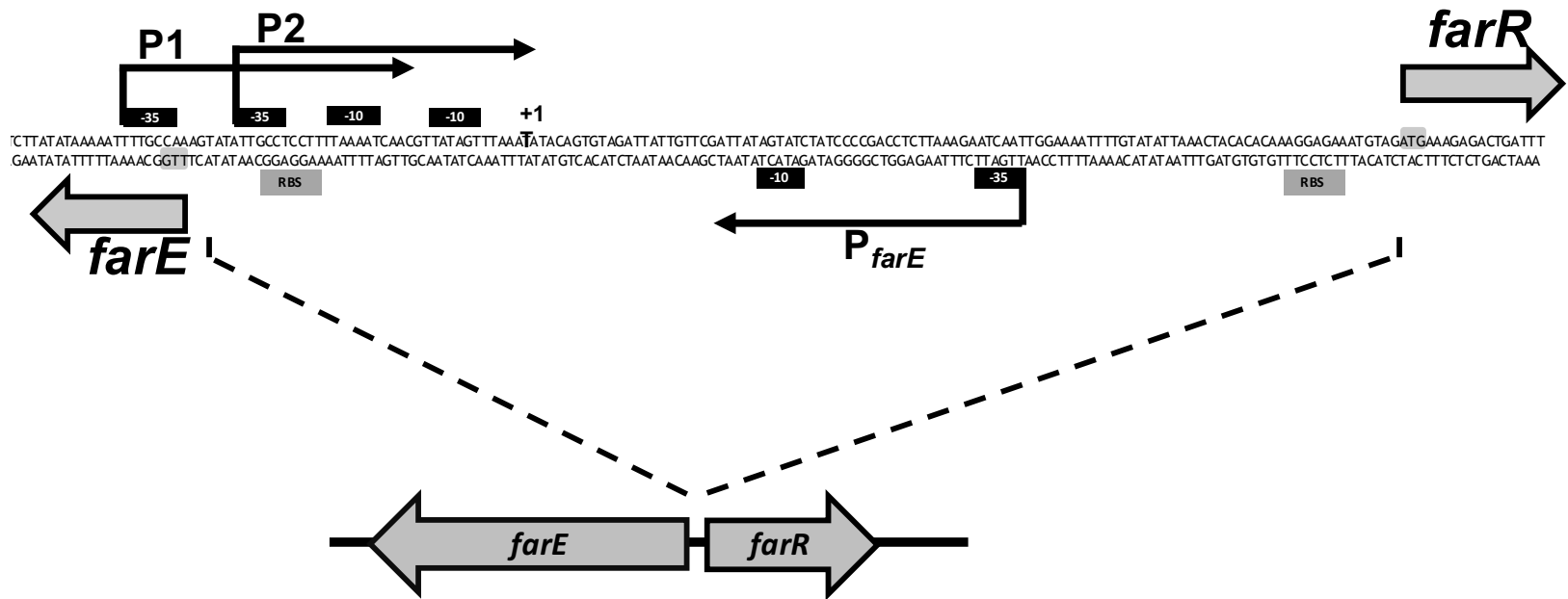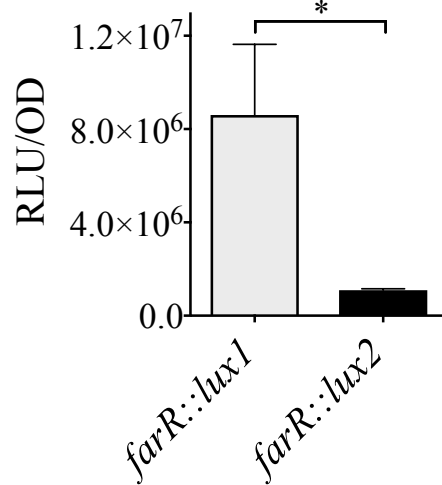
C. *farR::lux1* Activity in *S. aureus*

RLU/OD

4000

3000

2000

1000

0

USA300 USA300Δ*farER*

****
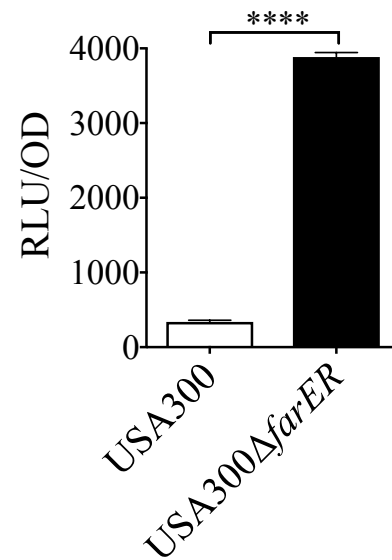
Figure 3.3 Nucleotide sequence of *farER* intergenic segment showing P$_{farR}$ and P$_{farE}$ promoter features (A), and *farR::lux* reporter gene assays conducted in *E. coli* (B) and *S. aureus* (C). The *farER* intergenic segment contains two potential P$_{farR}$ promoters, P1 and P2, and a predicted P$_{farE}$ promoter. The experimentally determined +1 transcription start site (TSS) of P2$_{farR}$ was confirmed using three independent 5′- RACE experiments. Translation initiation sites of *farE* (TTG) and *farR* (ATG) are in gray boxes. RBS (ribosomal binding site) of *farE* and *farR* are shown. **B**. Assay of *farR::lux* promoter constructs in *E. coli*. The larger *farR::lux1* construct contains both P1 and P2 promoters, while in *farR::lux2*, the P1 promoter is truncated within the predicted -35 promoter element. **C.** Assay of *farR::lux1* in *S. aureus* USA300 and USA300Δ*farER*. Each value represents the mean and standard deviation of three cultures, and each culture underwent quadruplicate luminescence readings at OD$_{600}$ of approximately 0.1 (**B**), and 0.5 (**C**). Results are shown normalized to the optical density. Statistical significance is determined using unpaired one-tailed Student's *t*-test, where *P <0.05 and ****P < 0.0001. Error bars represent Standard Error of the Mean.

3.3.5　Identification of FarR operator sites

Although the divergent arrangement of the *farE-farR* genes matches the classical paradigm of TFR function, in which the TFR serves to repress the divergently transcribed gene, our data indicate that FarR is needed to express FarE, rather than functioning as a repressor. However, as with a number of other TFR's, our data indicate that FarR represses its own expression. Therefore, we hypothesized that the *farER* intergenic segment should have two distinct operator sites to support binding of FarR; one to facilitate auto-repression of P$_{farR}$, and the other to function as an activator of P$_{farE}$. To identify potential operator sites, recombinant N-terminal 6×His-tagged FarR was expressed in *E. coli* and purified by metal affinity chromatography, for use in electrophoretic mobility shift assay (EMSA) experiments.

To determine if binding determinants are localized within the 145 bp *farER* intergenic segment, PCR with IRDye800 labelled primers was first employed to generate three ~ 160 bp probes that collectively encompass the entire intergenic segment, the 5′-end of *farR*, and the 5′-end of *farE* (OP$_{IN}$, OP$_R$, and OP$_E$, respectively). Using EMSAs, 6×His-FarR bound only to probe OP$_{IN}$ spanning the intergenic segment between the two genes, causing a mobility shift. This binding exhibited two protein-DNA complexes indicating that there are multiple binding sites of FarR within the intergenic segment (Fig. 3.4B). Since this confirmed that the primary FarR binding sites are contained entirely within the intergenic segment OP$_{IN}$, we proceeded to identify potential operator sites within this segment. TFRs bind palindromic, and often repeated, DNA operator sequences. Based on Pustell DNA matrix analysis of the 145 nucleotide intergenic segment that separates the translational starts of *farE* and *farR,* we identified a 17 nucleotide pseudo-palindrome containing three mismatches (PAL1), flanked by imperfect 16 nucleotide direct repeats (IR1 and IR2). These features span the transcriptional start site of the putative *farE* promoter region on the minus strand, and that of *farR* on the plus strand, thus comprising a likely site for binding of the FarR regulatory protein (Fig. 3.4A).

To evaluate the ability of these features to support FarR binding, we conducted EMSAs with 43mer probes centered on the intergenic segment; FarR bound to probe OP1 that encompasses the pseudo-palindrome as well as IR1 and IR2. FarR also bound OP2, which encompasses 6 nucleotides of 5′-end of the pseudo-palindrome, IR1 and structural elements of P*farR* as well as the +1 transcription start site of *farR,* but failed to bind to OP3 that contains 2 nucleotides of the 3′-end of the pseudo-palindrome, IR2 and structural elements of the putative P*farE* as well as the +1 transcription start site of *farE.* Additionally, the 52mer probe OP4, that spans the -35 element and additional sequence upstream of the putative P*farE*, also supported FarR binding (Fig. 3.4C). Interestingly, we identified a second pseudo-palindrome (PAL2) that bears similarity to PAL1 and is located in OP4 (Fig. 3.4A). PAL2 is a 16 nucleotide palindrome with four mismatches located 12 bp upstream of the -35 element of P*farE*. Therefore, there appears to be a FarR binding site centered on the structural elements of P*farR*, as well as another binding site upstream of the putative P*farE*.

To further refine these binding sites, we designed 28mer probes centered on OP1and OP2 (Fig. 3.4A). The probe OP5 is centered over the pseudo-palindrome, as well as 11 nucleotides of the 3′-end of IR1 and 8 nucleotides of the 5′-end of IR2. OP6 contains 9 nucleotides of the 5′-end of the pseudo-palindrome and IR1, whereas OP7 contains 8 nucleotides of the 3′-end of the pseudo-palindrome and IR2. FarR bound to OP5 and OP6, both of which span the +1 transcriptional start of *farR,* but did not exhibit any binding to OP7 (Fig. 3.4D).

The OP5 probe contains the entire PAL1, and to confirm specificity of protein-DNA interaction at this location, we employed competition EMSAs. In these assays, 50-fold excess of unlabeled competitor DNA is added to the reaction mix prior to incubation with the labeled probe. Specificity is confirmed by loss of binding to the labelled probe and thus lack of mobility shift. To this end, we designed a probe, OP5A, where the central GTGTAG sequence of the pseudo-palindrome was mutated to ACACCA and used it in a competition EMSA with the labelled OP5. Altering this central sequence of OP5 abolished the competition for FarR binding completely, confirming specificity of FarR binding to this operator site (Fig. 3.4E).

A.

B.

6XHis-FarR

OP$_E$ OP$_{IN}$ OP$_R$

← Free Probe

C.

6XHis-FarR

OP1   OP2   OP3   OP4

← FarR-Probe

← Free Probe

D.

6XHis-FarR

OP5   OP6   OP7

← FarR-Probe

← Free Probe

E.

6XHis-FarR

OP5 Without a competitor   Unlabelled OP5   Unlabelled OP5A

← FarR-Probe
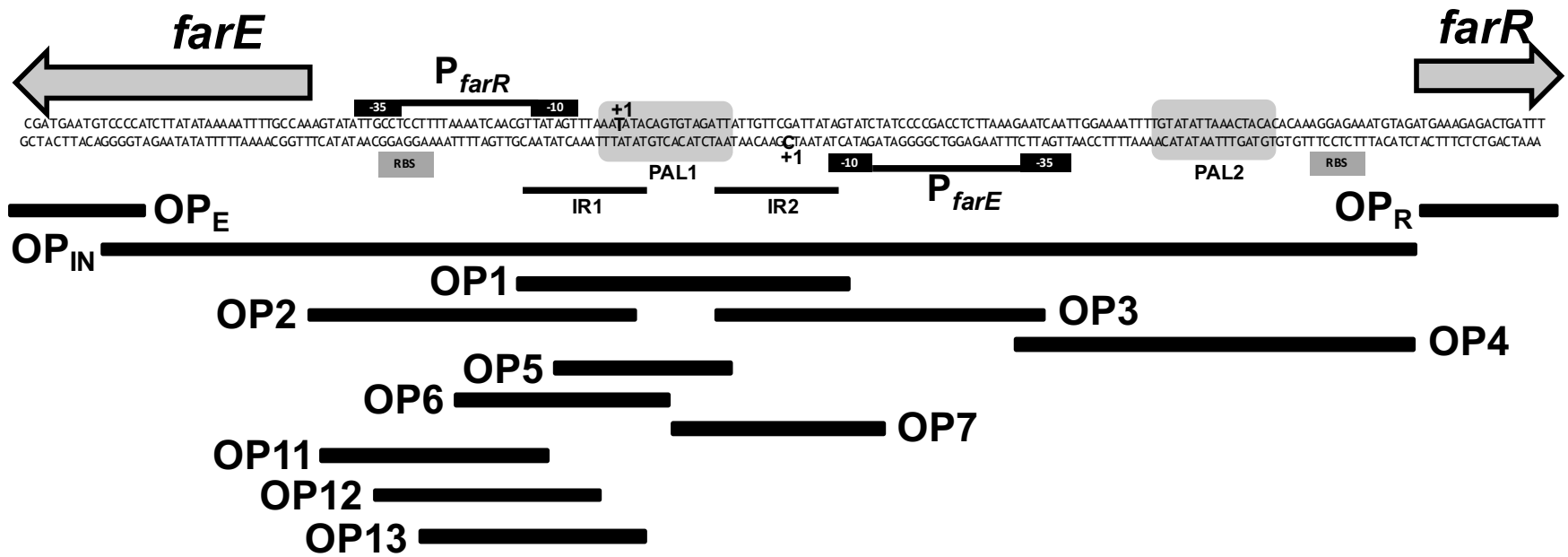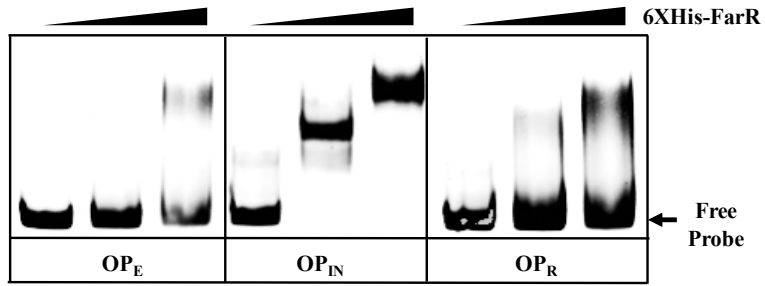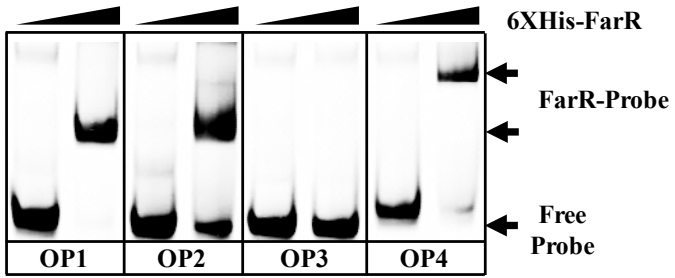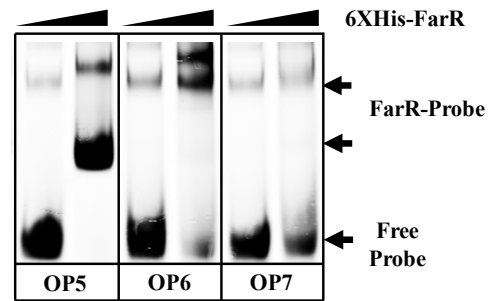
← Free Probe
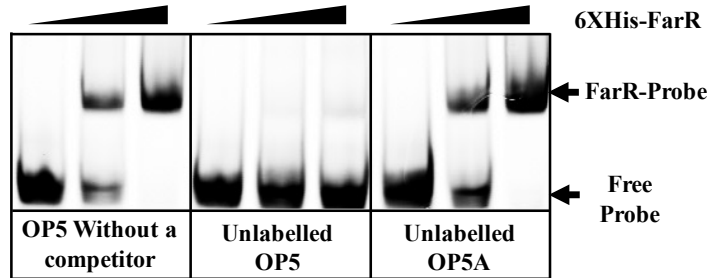
Figure 3.4 FarR operator sites are located in the *farER* intergenic segment. A. 145-nucleotide intergenic segment separates the translational initiation of *farE* and *farR*. Pseudo-palindromes PAL1 and PAL2 are in gray boxes. Inverted repeats IR1 and IR2 are underlined. Core promoter elements are indicated. Transcription start sites are in bold. For initial EMSA experiments with 6×His-FarR, PCR was employed to generate 167 bp IRDye-labelled probes OP_{IN}, OP_R, and OP_E that span the entire intergenic segment, the 5′-end of *farR,* and 5′-end of *farE,* respectively. The overlapping 43mers OP1, OP2, and OP3, and the 52mer OP4 collectively span the entire intergenic segment. The 28mers OP5, OP6 and OP7 and the 30mers OP11, OP12 and OP13 were designed to further narrow down the operator site that supports FarR binding. **B.** FarR binds operator OP_{IN} spanning the *farER* intergenic segment. In each reaction, 5 pmol of operator DNA is incubated with 0, 0.5 and 2 μM protein. **C. and D.** FarR binds to OP1, OP2, OP4, OP5 and OP6 and failed to bind OP3 and OP7. Each reaction contained 5 pmol of DNA with or without 2 μM protein. **E.** FarR binds to OP5 which spans the +1 transcription start site of *farR*, and nucleotide substitutions in PAL1 of this probe abrogates FarR binding. Competition EMSA experiments were done by incubating FarR with excess (50X) of unlabelled competitor probe for 30 minutes prior to incubating with 5 pmol of labelled probe.

3.3.6   FarR binds OP$_{Act}$ and OP$_{Rep}$

We have strong evidence that *farR* is required for *farE* induction (Fig. 3.2). Consequently, it appears that FarR does not function strictly as a transcriptional repressor, as is the case with most TFRs. There have been reports of two TFRs, LuxR in *Vibrio harveyi* and SczA in *Streptococcus pneumoniae*, that deviate from the norm of this family, where the protein functions as both repressor and activator depending on the presence or absence of a modulating ligand (13, 24, 25). This lead us to reason that FarR functions in a similar fashion where it binds a certain operator to function as a repressor and binds another operator site where it functions as an activator. Since we have evidence that FarR bound the operator OP4 located upstream of the putative P$_{farE}$, and that no other sequences upstream of P$_{farE}$ even within the 5′-end of *farR* supported protein binding, we speculated that binding to this operator would be most likely to activate *farE*. Additionally, FarR is subject to auto-regulation, and since operator probes OP2, OP5 and OP6 all support FarR binding, span P$_{farR}$ and immediately downstream of P$_{farE}$, we reasoned that binding to one of these operators would be to repress *farR*, and perhaps *farE*. To elucidate the minimal operator site that supports FarR binding for repression, we designed 30mer probes that span the structural features of P$_{farR}$, which is contained within the larger OP2 probe. OP11 spans 30 bp from the 5′-end of OP2, including the -35 element of P$_{farR}$. OP12 spans the center of OP2 and P$_{farR}$ including 4 bp of the -35 element and the entire -10 sequence of the promoter. OP13 contains 30 bp from the 3′-end of OP2, the -10 sequence of P$_{farR}$ as well as the transcriptional start site of *farR* (Fig. 3.4A). We performed competition EMSAs where 50-fold excess of these unlabelled competitor DNA probes are added prior to incubation with the labelled probe OP2. Both OP11 and OP12 were unable to compete with OP2, whereas OP13 competed effectively, and prevented protein binding to the labelled OP2, such that no mobility shift was observed (Fig. 3.5).
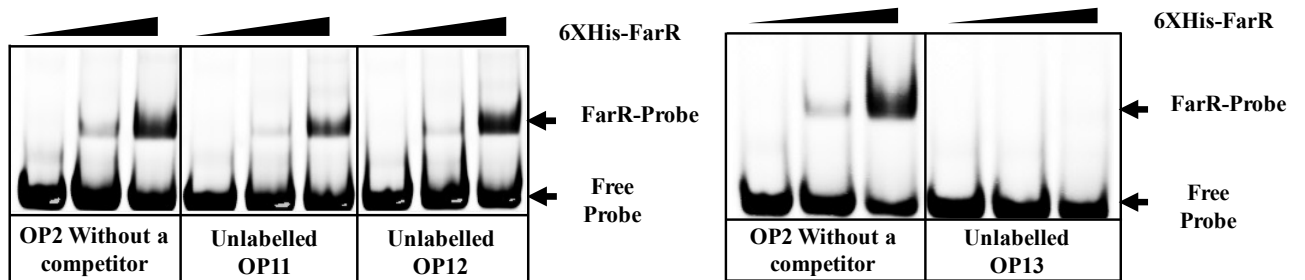
Figure 3.5 Competition EMSA with OP2 reveals that an operator site for binding of FarR is located within OP13. EMSA reactions contained 0, 0.5, or 2 μM 6×His-FarR, 250 pmol of unlabelled competitor, and 5 pmol of labelled OP2 probe. OP13 competes successfully with OP2 for binding of FarR, whereas OP11 and OP12 do not.

From these experiments, there appears to be two distinct operator sites for FarR binding in the intergenic segment between *farE* and *farR*. One operator is OP4, which contains sequences located immediately upstream of the putative P_{farE}. Since we established that *farR* does not function strictly as a repressor, we reasoned that this operator, hereafter termed OP_{Act}, is the site of FarR binding to enable activation of *farE*. Another distinct operator site spans the overlapping OP5 and OP13 probes. OP5 is centered over the PAL1 feature, which contains the +1 TSS of P_{farR} at its 5'-end, while its 3'-end is adjacent to the +1 TSS of P_{farE} on the minus strand. OP13 is centered on P_{farR}, and contains both the -10 promoter element and +1 TSS of P_{farR}. Since these two overlapping operator sequences span P_{farR} and transcriptional start site of *farR,* and immediately adjacent to that of *farE,* we reasoned that they represent repression operator site, OP_{Rep} (Fig. 3.6A).

3.3.7 OP_{Rep} contains the site of *farR* auto-repression and a $His_{121}Tyr$ substitution in FarR causes relief of auto-repression

Since *farR* is auto-regulatory, we reasoned that one of overlapping operators making up OP_{Rep} is the site of FarR binding to enable auto-repression. In previous work, we described a fatty acid resistant clone FAR7, which harbored a single nucleotide polymorphism causing a $His_{121}Tyr$ substitution in FarR. This clone exhibited increased constitutive levels of *farE*, even in the absence of fatty acid inducer, and displayed increased resistance to bactericidal concentrations of linoleic acid (1). When the reporter gene construct, *farR::lux,* was assayed in a FAR7 background we noticed a relief of the auto-repression when compared to wildtype USA300 (Fig. 3.6B). Therefore, it seems that the $His_{121}Tyr$ substitution, although not located within the N-terminal DNA binding domain of FarR, still results in less effective auto-repression. As such, we examined the ability of recombinant 6×His-tagged FarR protein of the variant FAR7 clone, hereafter termed FarR7, in mobility shift assays parallel to those done with the wildtype FarR protein. Interestingly, FarR7 was unable to bind to OP2, to which native FarR was able to bind efficiently (Fig. 3.6C).   OP2 spans P_{farR} and the +1 transcriptional start site of *farR*. To further define the operator sequence that supports differential binding of FarR and FarR7, we examined FarR7 binding to OP11, OP12 and OP13 that span the entire length of OP2. Strikingly, FarR7 did not exhibit any binding to OP13, and since FAR7

displayed a relief of auto-repression, these data suggest that OP13, which spans the -10 promoter element and +1 transcription start site of *farR,* also represents a site where FarR binds to mediate auto-repression (Fig. 3.6C).

A.

farE

farR

P*farR*

-35 -10

+1

PAL1

+1

-10 -35

P*farE*

PAL2

RBS

RBS

AATGTCCCCATCTTATATAAAAATTTTGCCAAAGTATATTGCCTCCTTTTAAAATCAACGTTTATAGTTTAAATATACAGTGTAGATTATTGTTCGATTATAGTATCTATCCCCGACCTCTTAAAGAATCAATTGGAAAATTTTGTATATTAAACTACACACAAAGGAGAAATGTAGATGAAAGAGACTGATTT

`TACAGGGGTAGAATATATTTTTAAAACGGTTTCATATAACGGAGGAAAATTTTAGTTGCAATATCAAATTTATATGTCACATCTAATAACAAGCTAATATCATAGATAGGGGCTGGAGAATTTCTTAGTTAACCTTTTAAAACATATAATTTGATGTGTGTTTCCTCTTTACATCTACTTTCTCTGACTAAA

IR1

IR2

OP5

OP13

OP4

OP**Act**

OP**Rep**

141

B.

farR::lux Activity in _S. aureus_

C.

Figure 3.6 FarR and the variant FarR7 differ in binding to OP2 and OP13, both of which span core promoter elements of P$_{farR}$. A. Mapping the oligonucleotide probes relative to core promoter elements of P$_{farR}$ and P$_{farE}$. Overlapping OP5 and OP13 that support FarR binding are grouped as OP$_{Rep}$. FarR also binds OP4 located upstream of P$_{farE}$, representing a potential activation site, OP$_{Act}$. **B.** *farR::lux* is de-repressed in the *S. aureus* FAR7 background. USA300 and FAR7 harboring the *farR::lux1* reporter construct were grown in TSB, and samples were withdrawn at OD$_{600}$ of approximately 0.5 for the determination of luciferase activity. Each data point represents the mean and standard deviation of three cultures, and each culture sample was subjected to quadruplicate luminescence readings at OD$_{600}$. Statistical significance is determined using unpaired one-tailed Student's *t*-test, where **P <0.01. Error bars represent Standard Error of the Mean. **C.** Mobility shift assay showing that FarR7 does not exhibit any binding to OP2 or OP13. In each reaction, 5 pmol of operator DNA is incubated with up to 2 μM protein.

3.3.8   Specificity determinants of FarR binding

Aside from the sequence similarities between PAL1 and PAL2, the OP4, OP5 and OP13 probes that comprise OP_{Rep} and OP_{Act} appear to share no obvious sequence similarities. However, EMSA experiments confirmed that OP4, OP5 and OP13 can cross-compete for FarR binding and that nucleotide substitutions in any of them abrogated this competition completely. Interestingly, all three operators contained a TAG sequence central to each operator (Fig. 3.7A).   To determine if this represents a specificity determinant for protein binding, we performed a competition EMSA with probes containing nucleotide substitutions (OP5TA**G**>**A**, OP13TA**G**>**A**) or deletion (OP5Δ**TAG**) in this sequence and found that these mutations eliminated FarR binding (Fig. 3.7B). Furthermore, analysis of multiple sequence alignment of 13 staphylococcal species that contain divergent *farER* revealed that FarR specificity determinants are conserved among these staphylococci. Importantly, the TAG nucleotides located in OP4, OP5, and OP13 are highly conversed among these species (Fig. 3.8).

Interestingly, in OP13, the TAG nucleotides are overlapping with the -10 element of P_{farR} and this operator is the only operator that exhibited differential binding of FarR and FarR7. Additionally, our reporter gene assays revealed that *farR::lux* is de-repressed in the FAR7 background, thus providing further evidence that OP13 is the site of FarR auto-regulation. To correlate these data with *in vitro* analyses, we introduced the same substitution in OP13 (OP13TA**G**>**A** where the -10 element of P_{farR} is mutated) into the existing reporter gene construct, *farR::lux* by site-directed mutagenesis. When we assayed the mutated $farR_{TAG>A}$*::lux* luciferase activity in USA300, there was a relief of auto-repression similar to that seen in the USA300Δ*farER* and FAR7 backgrounds, confirming that OP13 is indeed the site of auto-repression of *farR* (Fig. 3.9A and B). We also prepared cell lysate fractions from *E. coli* complemented with pLI50 harboring native *farR* and *farR* bearing mutated P_{farR} grown in LB and showed by western blotting that mutating P_{farR} indeed relieves auto-repression, and promotes FarR expression (Fig. 3.9C).

A.

OP4
$3'$ TAAAGAGGAAACACACATCAAATTATATGTTTTAAAAGGTT $5'$
$5'$ ATTTCTCCTTTGTGTG**TAG**TTTAATATACAAAATTTTCCAA $3'$

OP5
$5'$ AGTTTAAATATACAGTG**TAG**ATTATTGTT $3'$
$3'$ TCAAATTTATATGTCACATCTAATAACAA$5'$

-10   **+1**

OP13
$5'$ TTTTAAAATCAACGT*TA**TAG***TTTAAATATA $3'$
$3'$ AAAATTTTAGTTGCAATATCAAATTTATAT$5'$

B.
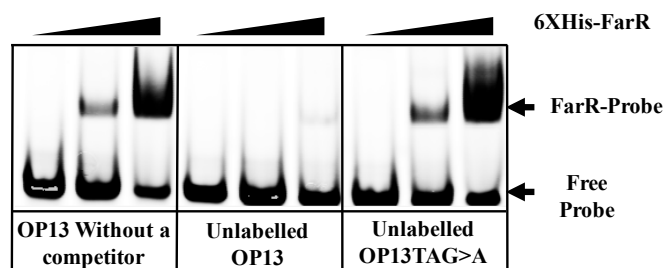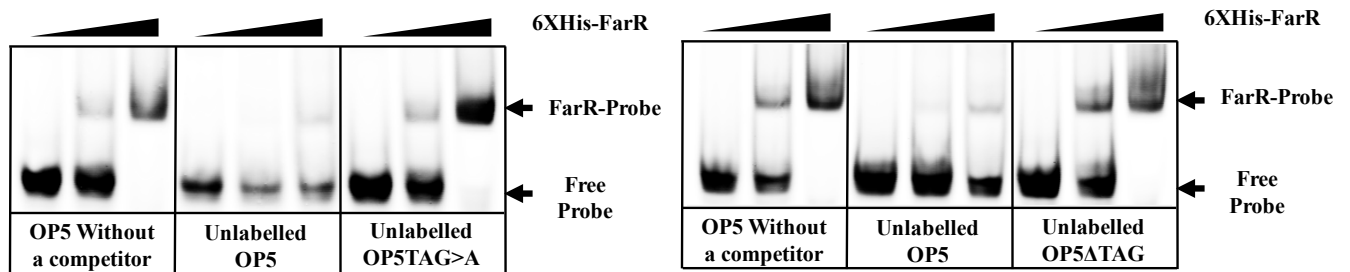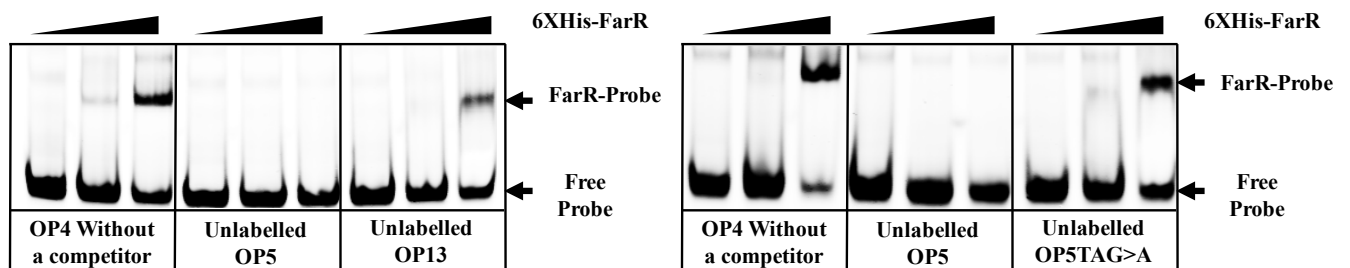
Figure 3.7 OP4, OP5 and OP13 cross-compete with each other and nucleotide substitutions obliterate this competition. A. Sequence similarities shared between OP4, OP5 and OP13. Arrows indicate the orientation of the similar sequence. The +1 TSS of *farR* is labelled +1 in OP5 and OP13, and nucleotides comprising the -10 promoter element of P*farR* are italicized in OP13. **B.** OP4, OP5 and OP13 cross-compete with each other. Nucleotide substitutions in OP5 and OP13 (TA**G**>**A**) prevented this competition with OP4, confirming that this site is a specificity determinant for FarR binding. Each reaction contained 0, 0.5, and 2 μM FarR mixed with 250 pmol of unlabelled competitor and 5 pmol of labelled probe.

```
     USA300        1 CAAAGTATATT-GCCTCCTTTTAAAATCAACGTTATAGTTTAAATATACAGTGTAGATTATTG---------------TTCGA-TTATAGTA----TCTATCCCCGACCTCTTAAAGAATCAATTGGAAAATTTTGTATATT-AAACTACACACAAAGG 137
S. equorum       1  CAAACTATT-GCCTCCTAATTTAATCAACGTTATAGTTTATACATATAGTGTAGATTATTT--------------ATATTA-ACATTTTACAA-GCTAAGTCGTATTATAAAAAGAACTAATTATGCCAATATGTCTATTTAAACTACAAAA-GGG 139
S. xylosus       1  CAAACTATT-GCCTCCTAATTTAATCAACGTTATAGTTTAAATATATACTGTAGATTATTT--------------CTATCC-ACATATTA-AATGCTTTACCCTTTTATTAAAAGAATTAATTATTACAATTTGTATATTTAAACTACAAATA-AGG 139
S. argenteus     1  CAAAGTATT-GCCTCCTTTTAAAATCAACGTTATAGTTTAACTATACAGTGTAGATTATTG--------------TTAGA-TTATATTA----TCTATACCCTACCTCTTAAAGAATTAAATTTTAAAATTTGTATACT-AAACTACACATTAAGG 135
S. saprophyticus 1  CAATCTATT-GCCTCCTCAGATAATCAACGTTATAGTTTAATTGTATAGTGTAGATTATTT--------------ATATTA-ACACAATA-AACGCTGTGTACAATTAATAAAAGAATTAAAAACTGTAATATGCCTATTTAAACTACAGATT-AGG 139
S. carnosus      1  CAAAGTATT-GCCTCCTCCTAAAATATACAGTATAGTTTA-TTCTACATAGTGTAGATTAATTAC-----------GTATCACATATGCTA-CATGGTTAATTATTACCTGTACAGATATATTTCAAGCCTAATATAAGGCTAAACTACACTAA-GGA 141
S. cohnii        1  CAAACTATT-GCCTCCTTAATTAATCAACGTTATAGTTTATACATACTGTAGATTATTT--------------CTATTG-ACATCTTATAATGATTTACCCTATTTAAAAAAGAACTAAATATCCAATTCCGTATATTTAAACTACAGATT-AGG 140
S. succinus      1  CAAACTATT-GCCTCCTTATTTAATCAACGTTATAGTTTAAATGTAATGTGTAGATTATCT--------------ATAATA-TCATAATAACCATGCTCCCCCTAATATATAAAGAATTAAATATACTAAAATGTATATTTAAACTACAAATA-AGG 140
S. schweizeri    1  CAAATATATT-GCCTCCCTTTAAAATCAACGTTATAGTTTAACTATACAGTGTAGATTATTG--------------TTCGA-TTATAGTA----TCTATACCCTACCTCTTAAAGAATTAAATTTTAAAATTTGTATACT-AAACTACACATTAAGG 136
S. simiae        1  CAAAGTATT-GCCTCCTCTTGATTTCAACGCTATAGTTTAACTGTATAGTGTAGATTATTG--------------TGTGA-TTATAATA----TCTCTACCCTCTTTTTAAAAGAAATAAATAATCGATTGTGTATTTT-AAACTACATATTA-GG 134
S. gallinarum    1  CAAACTATTTGCCTCCCTATTTAATCAACGTTATATTTTAAATATATTATGTAGATTATCTGTATCTTACATATTCATAGTA-ACATACTAATCAT-TTTTGATTCTTTTATAAAGAATTAATAATATAGAACTGTATATTTAAACTACACATA-AGG 155
S. arlettae      1  CAAGCTATT-GCCTCCTTATTTAATCAACGTTATAGTCTAAATATATAGTGTAGATTATTT--------------ACAGTA-CTATACTACTAGTAATTCCCCCAATTT-TAAAGAAATAATAATTATTATATGTATATTTAAACTACAAAAACAGG 140
S. lugdunensis   1  CAAAGTATT-GCCCCCTTCTAAAATCAACGTTATAGTTTAACTATACATTGTAGATTATTTAA-----------CATGCCA-ATATGTTA-CATTCTTTTGCTCTGTATTTAAAGTCATAATTAATGATATATGTATAGATAAACTACACGGTTTGGAG 143
                    *   **** *** **        *  ** **** * **     **   ********               * **         * **    *  * **   *                                       *******
```

Figure 3.8 Multiple sequence alignment of the intergenic segment between *farE* and *farR* among *S. aureus* USA300 and 12 other staphylococcal species that contain divergent *farER*. The alignment was generated using ClustalW 1.4. Underlined sequences represent FarR binding motifs in OP13; OP5; and OP4, respectively. Bold and highlighted nucleotides indicate FarR favored nucleotides at positions 4,5 and 6 of each binding motif.

A.

farR::lux Activity in USA300

□■ *farR::lux*;TSB
○● *farR*TAG>A::*lux*;TSB

B.

farR::lux Activity in different backgrounds


□ *farR::lux*
■ *farR*TAG>A::*lux*

C.

Figure 3.9 Nucleotide substitution in the -10 element of P_{farR} causes a relief of auto-repression. A. Growth (OD_{600}; open symbols) and relative luminescence units (RLU/OD; closed symbols) of USA300 harbouring the pGY*farR::lux* and pGY*farR*$_{TAG>A}$*::lux* reporter construct are shown. **B.** Assay of *farR:lux* and *farR*$_{TAG>A}$*::lux* in different genetic backgrounds. *S. aureus* USA300 harboring either pGY*farR::lux*, or pGY*farR*$_{TAG>A}$*::lux* were grown in TSB to an OD_{600} of approximately 0.5, and cultures were processed for determination of luciferase activity. For comparative purposes, assays were also conducted on cultures of *S. aureus* FAR7, and USA300Δ*farER* harboring native pGY*farR::lux*. Each value represents the mean and standard deviation of three cultures and each culture underwent quadruplicate luminescence readings at each time point (**A**) or at OD_{600} of approximately 0.5 (**B**). Error bars represent Standard Error of the Mean. **C.** Western blot for detection of FarR protein in cell lysates of *E. coli* complemented with pLI50, pLI*farR* or pLI*farR*$_{TAG>A}$. Each lane contains 25 µg of total cell lysate protein, and the control lane contains 5 ng of recombinant 6×His-FarR.
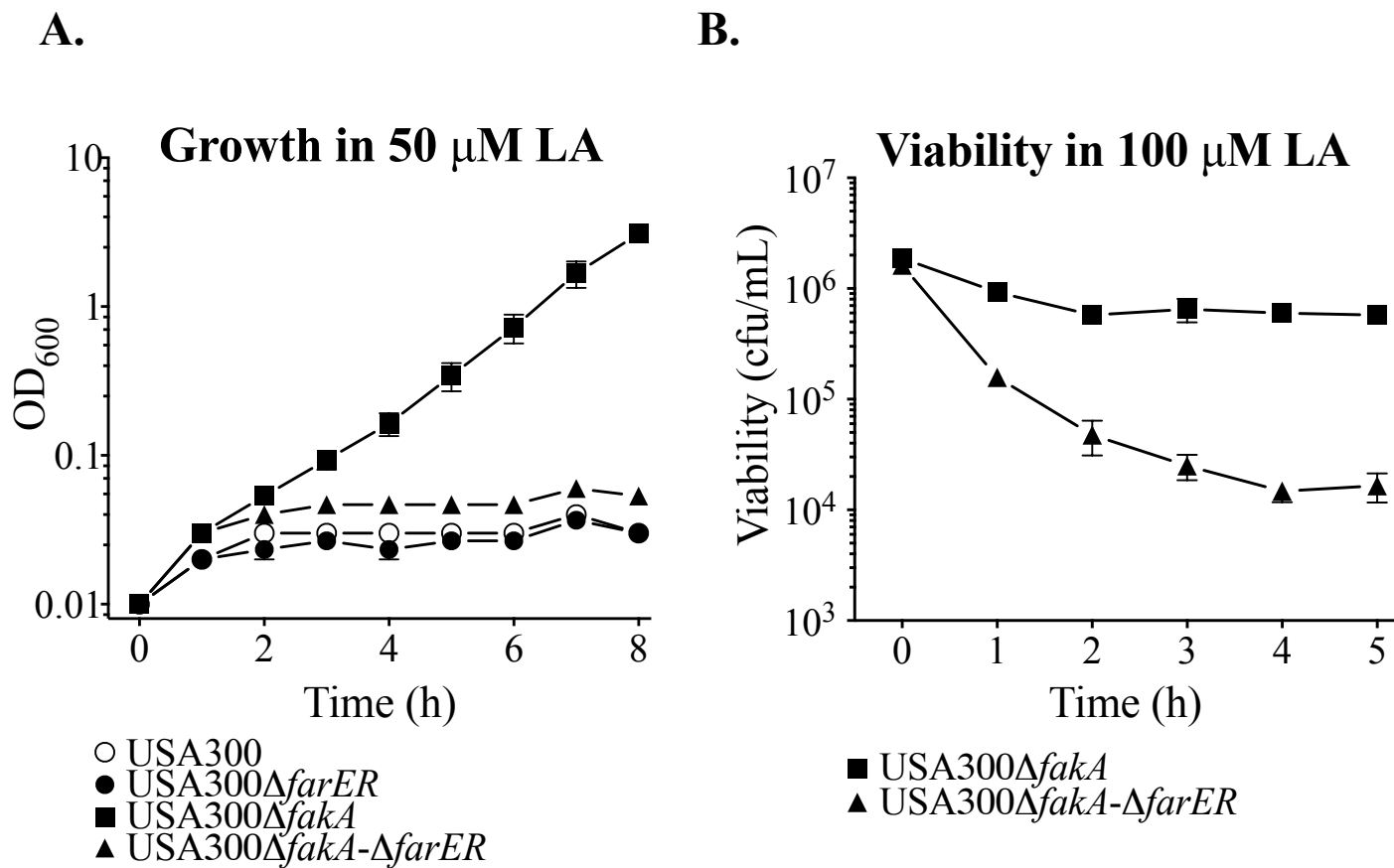
3.3.9 *farER* and fatty acid detoxification

Since *S. aureus* is incapable of β-oxidation of fatty acids, the metabolic fate of exogenous unsaturated fatty acids is incorporation into the phospholipid component of the membrane (26). Parson *et al.* identified the fatty acid kinase system that is responsible for processing the exogenous fatty acids that enter the cell. FakB1 and FakB2 bind saturated and unsaturated fatty acids, respectively. FakA then phosphorylates the fatty acids and the resulting acyl-PO_4 is either extended by FASII machinery or directly incorporated into phospholipid (27). Importantly, TFRs are typically responsive to small hydrophobic ligands and crystallography studies of these regulators, including the fatty acid degradation regulator FadR of *Bacillus subtilis*, have revealed that endogenous fatty acid derivatives such as acyl-coAs are often co-crystallized with the protein from the *E. coli* host (28). We reasoned that phosphorylated fatty acids could represent the physiologic ligand that modulates FarR function and interaction with its operator sites. Therefore, we assayed the reporter gene construct, *farE::lux* in USA300Δ*fakA*, to examine *farE* expression when the ability to phosphorylate fatty acids is abrogated. Interestingly, USA300Δ*fakA* exhibited increased constitutive levels of *farE,* but expression could not be further induced by exogenous linoleic acid (Fig. 3.10). Further, this mutant was also significantly more resistant to killing by bactericidal concentrations of linoleic acid compared to wildtype USA300, and this phenotype is *farE*-dependent (Fig. 3.11).
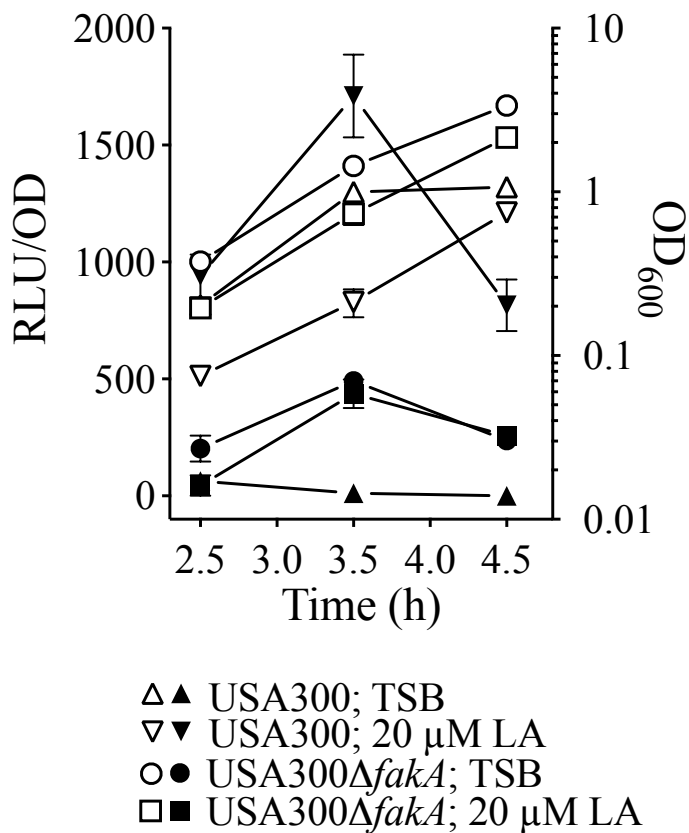
Figure 3.10 *farE::lux* **exhibits elevated constitutive levels of expression in the absence of fatty acid kinase,** *fakA***.** Growth (OD_{600}; open symbols) and relative luminescence units (RLU/OD; closed symbols) of USA300 and USA300Δ*fakA* harbouring the pGY*farE::lux* reporter construct are shown. Strains were grown in TSB or TSB supplemented with 20 μM linoleic acid (LA). Each value represents the mean and standard deviation of three separate cultures, and each culture was subjected to quadruplicate luminescence readings at each time point. Error bars represent Standard Error of the Mean.

A.

Growth in 50 μM LA

○ USA300
● USA300Δ*farER*
■ USA300Δ*fakA*
▲ USA300Δ*fakA*-Δ*farER*

B.

Viability in 100 μM LA

■ USA300Δ*fakA*
▲ USA300Δ*fakA*-Δ*farER*

Figure 3.11 USA300Δ*fakA* exhibits enhanced resistance to linoleic acid, in a *farE*-dependent manner. A. Strains were grown in 50 μM linoleic acid. **B.** Strains were grown to mid-exponential phase in 20 μM linoleic acid, and then diluted to 10^6 cfu/mL in fresh TSB containing 100 μM linoleic acid. Viable cell counts were taken at hourly intervals. Each data point represents mean value of triplicate (**A**) or quadruplicate (**B**) cultures. Error bars represent Standard Error of the Mean.

3.3.10 *farER* influence virulence in a subcutaneous abscess infection model

Studies on staphylococcal murine abscesses reveal that antibacterial activity dwells in the free fatty acid fraction of the abscesses (29). NorD, a *S. aureus* multidrug efflux pump from the major facilitator superfamily, was upregulated during infection and played a part in the bacterial fitness in an infection abscess model (30). Similarly, NorB in the *S. aureus* MW2 strain bestows fitness advantages in the murine subcutaneous abscess model. The pattern of expression of *nor* and *tet38* pumps in murine abscess models was found to be distinct from that observed during *in vitro* cell culture, and this was reasoned to be due to environmental triggers that dictate the cellular response (31). The fatty acid kinase, *fakA,* has also been linked to virulence in a murine model of *S. aureus* dermonecrosis; *fakA*-deficient mutants were more pathogenic than wildtype and formed larger abscesses (32). To assess the contribution of *farER*-mediated fatty acid resistance to virulence *in vivo*, and link it to the hyper-virulent phenotype observed in a *fakA*-deficient mutant, we constructed a USA300Δ*fakA*-Δ*farER* double mutant and utilized it in a murine abscess infection model. In agreement with a previous report, USA300Δ*fakA* exhibited a trend towards increased surface abscess area compared to wild type USA300, but this enhanced virulence phenotype was abrogated in USA300Δ*fakA*-Δ*farER* (Fig. 3.12). In contrast, the single mutant, USA300Δ*farER,* showed no difference in virulence compared to that of wildtype USA300 (Appendix 7). Therefore, the increased basal level of *farE* expression in USA300Δ*fakA* appears to promote increased resistance to antimicrobial fatty acids *in vitro*, and enhanced virulence in a subcutaneous abscess infection model.

A.

B.
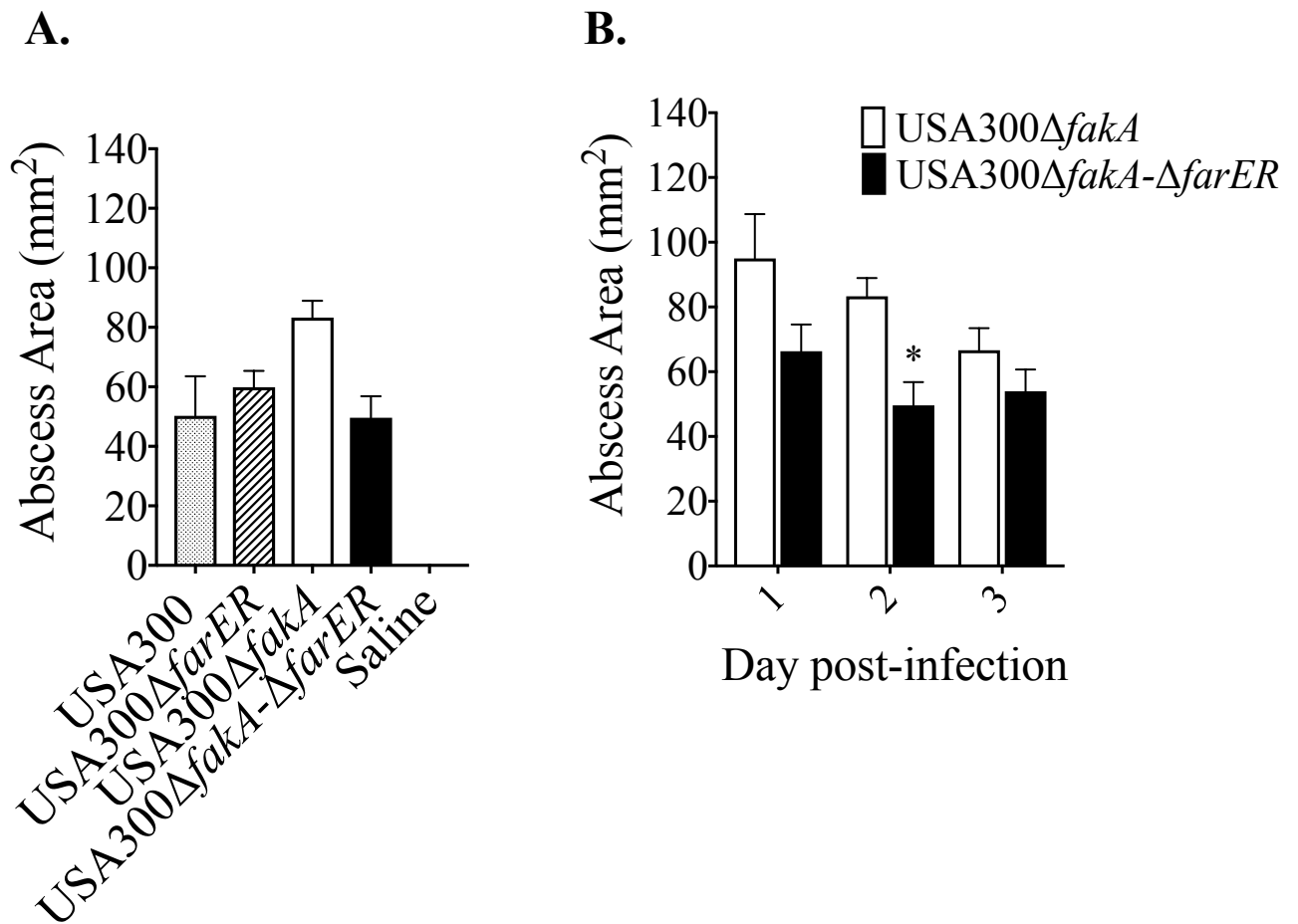
Figure 3.12 USA300Δ*fakA-farER* exhibits reduced virulence in a murine skin abscess model of infection. 6-week female BALB/c mice were infected with 2×10^7 CFU of USA300, USA300Δ*farER*, USA300Δ*fakA* and USA300Δ*fakA-farER*. Assesses were imaged daily and surface area was quantified using ImageJ. **A.** Abscess surface area at 2 days post-infection. **B.** The virulence exhibited by USA300Δ*fakA* was in a *farER*-dependent manner consistently throughout the course of infection. The reduction in virulence exhibited by USA300Δ*fakA-farER* is significant relative to that of USA300Δ*fakA* as determined by a two-way ANOVA where *$P < 0.05$. Error bars represent Standard Error of the Mean. n=8 abscesses per strain.

3.4 Discussion

This work provides the first mechanistic insight into the regulation of the RND family efflux pump FarE, by the TFR FarR, which together function to confer resistance of *S. aureus* to antimicrobial unsaturated free fatty acids. FarR, as with other TFRs, is auto-regulatory in nature. The transcription of *farE* increases in response to linoleic and arachidonic acids and gene fusions demonstrated that expression of *farE* is induced only in the presence of functional FarR. This represents one of the very few TFRs with dual functionality. 6×His-tagged FarR binds an operator overlapping the -10 element of P*farR*, upstream of the transcriptional start site of the gene, as well as another operator downstream of the transcriptional start site. 6×His-tagged FarR also binds a third operator upstream of P*farE*. Taken together, our mobility shift and reporter-gene assays establish a working model for us to further refine the mechanism by which *farE* is regulated by *farR*. First, FarR binds to an operator site within OP13, which includes the -10 motif of P*farR*, leading to strong repression of FarR expression. An overlapping but independent operator site within OP5 could potentially function to repress both *farR* and *farE*, while binding to a third site in OP4 which is upstream of the predicted P*farE*, is most likely to promote activation of *farE* expression. For simplicity, we refer to OP13 and OP5 as OP$_{Rep}$ and OP4 as OP$_{Act}$.

Reporter-gene assays show that FarR has a repressive effect on its own gene, independent of the addition of fatty acid ligand, as evident from the low activity of *farR::lux* during growth of USA300 in TSB. Consequently, during growth in TSB, our data indicate that *farR* is strongly auto-repressed. Accordingly, the introduction of a single base pair substitution in the -10 element of P*farR* led to abolishment of FarR binding in mobility shift experiments, and relief of auto-repression in reporter-gene assays. Similarly, a single nucleotide polymorphism that resulted in a His$_{121}$Tyr substitution in FarR displayed a constitutive level of *farE* expression in the absence of a fatty acid inducer, concomitant with increased resistance to linoleic acid, and less effective repression of *farR*. Therefore, to identify the operator site responsible for auto-regulation, we looked for differential binding between FarR and the FarR7 protein. Although OP5 partially overlaps with OP13, it supported FarR7 binding, whereas OP13 did not, suggesting that the latter is the

dedicated site for auto-repression. Although the location of this $His_{121}Tyr$ substitution was not within the N-terminal DNA binding domain of FarR, our data suggest that it does have a role in maintaining DNA-binding affinity. In a similar example, certain residues of the C-terminal ligand domain of *B. subtilis* FadR undergo structural changes upon acyl-CoA binding, to affect the conformation of the DNA-binding domain. Mutations in these residues resulted in lower DNA binding affinity (28).

Since 6×His-tagged FarR also binds OP5, it is feasible that OP5 is also a site for *farE* repression. Typical repressors such as the prototypic TetR bind inverted repeats located downstream or spanning the -10 element of their respective promoters (28), and OP5 is located immediately downstream from the putative +1 transcription start site of *farE*. Although binding to OP5 may not prevent RNA polymerase binding as anticipated of typical transcriptional repressors, it would still obstruct transition of the transcription complex, causing repression. Similarly, the staphylococcal QacR, a repressor of multidrug efflux pump QacA, binds an inverted repeat located immediately downstream of the *qacA* promoter. This operator also overlaps the transcription start site of *qacA* to block the transition of the RNA polymerase-promoter complex that otherwise allows for gene transcription (34, 35). Additionally, RutR, a TFR in *E. coli*, represses its own expression by binding to an operator site located downstream of its transcription start site. This binding mode seems to be indicative of negative regulation by either interfering with open complex formation, elongation complex initiation, or RNA polymerase transition (36, 37). Conversely, both FarR and FarR7 bind OP4, located upstream of P*farE*, equally well, and do not bind any other operators upstream of P*farE* or within the 5′-end of the divergent *farR*, suggesting that binding to OP4 would be most likely to activate *farE*.

Studies show that the activation of the *acrAB* promoter in *E. coli* is in response to accumulation of cellular metabolites, and that global transcriptional activators (MarA and SoxS) and a local TetR-transcriptional repressor (AcrR) regulate the RND-efflux pump AcrB, to ensure that this pump is only expressed when needed. Binding of a wide range of toxic compounds to the C-terminal ligand binding domain of AcrR results in a conformational change in its N-terminal DNA binding domain, and this in turn releases the protein from operator DNA and permits transcription. However, the inactivation of

AcrR must also be accompanied by the induction of global regulators in order to achieve *acrAB* expression (8, 7, 38). Consequently, relief of de-repression alone is insufficient to promote constitutive expression of the AcrB efflux pump. In the context of FarE and FarR, binding of FarR to both OP_{Rep} and OP_{Act} may be a regulatory mechanism by which FarR controls the expression of the *farE* efflux pump to ensure it is only expressed when needed. We also examined all three operators that supported FarR binding and attempted to identify any common sequence that may be a specificity determinant for protein binding. There seems to be a clear preference for a common **TAG**(T/A)TTA binding motif in all three operators; 5′GTG**TAG**TTTAAT3′ in OP4, 5′GTG**TAG**ATTATT3′ in OP5, and 5′TTA**TAG**TTTAAA3′ in OP13. We performed competition EMSAs with a combination of mutations and deletions of these motifs and found that FarR discriminates in favor of **TAG** nucleotides at positions 4, 5 and 6 of each binding motif. Interestingly, analysis of multiple sequence alignment of 13 staphylococcal species that contain divergent *farER* revealed that FarR binding motifs are conserved among these staphylococci. Importantly, the TAG nucleotides located in the binding motifs of OP4, OP5, and OP13 are highly conversed among these species (Fig. 3.8).

Structural studies will be needed to further shed light on the exact regulatory mechanism of FarR and its mechanism of de-repression and *farE* activation. There have been reports of TFRs with unconventional regulatory functions, such as RutR, that can both activate and repress gene expression in its apo-form. RutR is auto-regulatory, represses a divergently transcribed gene, and activates yet a third promoter. The mechanism by which RutR activates gene expression is yet to be determined, but the location of its operator sites suggests a mechanism of de-repression rather than actual recruitment of transcription machinery (36, 39). Another unconventional TFR is AmtR of *Mycobacterium smegmatis* which is involved in a post transcriptional regulation of urea metabolism without binding to any effector molecule. This regulation utilizes a sensory system that involves a trans-acting sRNA in response to nitrogen availability. When nitrogen is overabundant, the sRNA targets *amtR* mRNA and blocks its translation. Under nitrogen starvation, the sRNA is down-regulated and *amtR* is successfully translated. The AmtR repressor then works in combination with the global nitrogen

activator, GlnR, to fine-tune the expression of urea-degrading metabolic pathway (40). Another possibility is that FarR exhibits a regulatory network similar to that of DhaS, an unconventional TFR from *Lactococcus lactis* that functions as an activator. Similar to FarR, DhaS binds an operator site upstream of the Dha promoter, spanning the -35 element of the promoter. The mode of action of DhaS is not a direct de-repression, but rather involves a transcription co-activator, DhaQ, that forms a complex with DhaS upon ligand, dihydroxyacetone, binding and subsequently activates transcription of the dihydroxyacetone operon (12).

Upon entry into the cell, exogenous fatty acids bind FakB, and after phosphorylation by FakA, the resulting acyl-PO$_4$ is incorporated into phospholipid directly, or alternately, the PO$_4$ is exchanged for a CoA moiety, and the fatty acid passes through an extension cycle by the FASII machinery. A recent report by Lopez *et al.* has demonstrated that a Type VII secretion system (T7SS), which is crucial for prolonged bacterial survival and persistence of *S. aureus* in abscesses, is activated by uFFAs such as linoleic and arachidonic acids. Additionally, this activation is contingent on the incorporation of uFFAs into phospholipids and lipoprotein by the Fak machinery (41, 42). Therefore, the similar virulence we observe in wildtype USA300 and USA300Δ*farER* is perhaps due to functional Fak machinery. Despite the evidence of a connection between *farER* and the fatty acid kinase *fakA in vitro* and *in vivo*, a direct interaction between FarE, FarR and FakA has yet to be demonstrated. Here we show that USA300Δ*fakA* exhibits increased constitutive levels of *farE*, but expression cannot be further induced by exogenous linoleic acid. This mutant was also significantly more resistant to killing by bactericidal concentrations of linoleic acid compared to wildtype USA300, and this phenotype was *farE*-dependent. A recent report indicates that USA300Δ*fakA* exhibited an elevated pool of intracellular fatty acids (43). Consequently, as reported in other RND efflux systems, an increased pool of intracellular metabolites in USA300Δ*fakA* may be responsible for increased basal expression of *farE*. Interestingly, FarR binding to OP$_{Rep}$ was partially reduced in the presence of linoleoyl-CoA and arachidonoyl-CoA (Fig. 3.13). Therefore, perhaps the accumulation of intracellular fatty acids results in the *fakA* mutant background leads to partial de-repression of *farE*, which cannot be fully induced due to

the fatty acids being unable to be phosphorylated in the absence of *fakA*. As such, it appears likely that phosphorylated fatty acids are the signal for regulation of *farE* via *farR*. Experiments to test this would contribute to our understanding of the complex *farR*-dependent activation of *farE*. Antimicrobial unsaturated fatty acids represent a vital component of innate immunity, and *S. aureus* is constantly exposed to them, not only during colonization but also in the context of an infection. It is evident that one of the strategies *S. aureus* employs to cope with these fatty acids is via a complex regulatory mechanism where a TFR with dual functionality controls the expression of a fatty acid specific efflux pump.
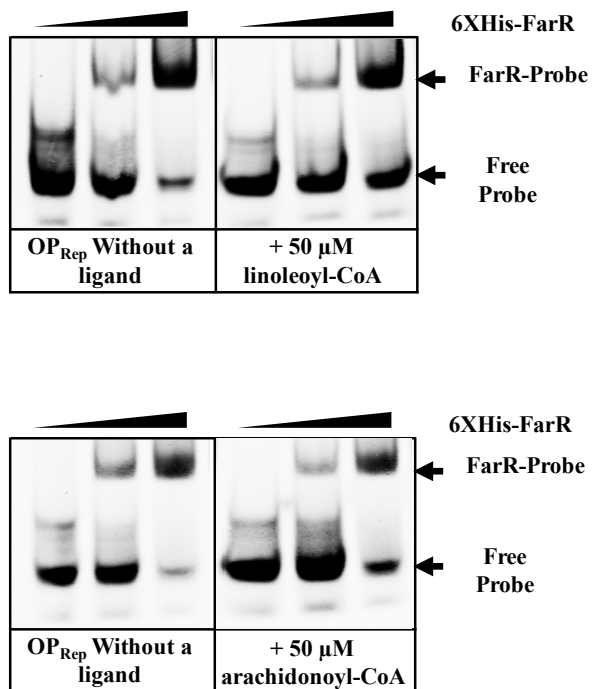
Figure 3.13 FarR binding to OP_{Rep} is partially affected by linoleoyl-CoA and arachidonoyl-CoA. In each EMSA reaction, 5 pmol of DNA is incubated with 0, 0.5 or 2 μM FarR in the presence or absence of 50 μM ligand, as indicated.

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Chapter 4

4   General Discussion and Conclusions

4.1 Summary

S. aureus USA300 is the current epidemic strain of CA-MRSA in North America. It accounts for 98% of MRSA infections presented to emergency departments in the United States. This strain is notorious for its ability to overproduce toxins and virulence factors, as well as its remarkable ability to persist on skin surfaces. In order to persist on skin surfaces, the bacterium must have intrinsic mechanisms to cope with the antimicrobial uFFAs that constitutes an important component of the immune defense mechanisms on the skin. These uFFAs are membrane-disruptive, and humans deficient in the production of these fatty acids are more susceptible to *S. aureus* infections. The purpose of this thesis was to better understand the genetic basis for *S. aureus* USA300 adaptation to uFFAs which allows this strain to establish a successful infection. When USA300 variants were selected for their ability to grow in elevated levels of uFFAs, two out of the seven clones that were sent for whole genome sequencing contained a SNP in an uncharacterized gene, *SAUSA300_2490*. Using domain enhanced lookup time accelerated BLAST, the amino acid sequence of *SAUSA300_2490* has 99% homology to TFRs. Furthermore, structure prediction using homology modeling server, Phyre2, predicts that *SAUSA300_2490* shares 99.8% amino acid sequence similarity with FadR, a TFR of fatty acid degradation in *T. thermophilus*. Typically, TFRs exert their effects on divergent genes. Bioinformatics revealed that the *SAUSA300_2489* gene, which is divergently transcribed from *SAUSA300_2490*, encodes a gene product that belongs to the Resistance-Nodulation-Cell Division (RND) superfamily of proteins, which promote proton-antiport dependent efflux mechanisms. The *SAUSA300_2489* gene has an MMPL domain that codes for members of putative integral transmembrane proteins that are linked with lipid-metabolizing enzymes, suggesting a possible role in the efflux of fatty acids. As such, in chapter 2 my aim was to characterize *SAUSA300_2490* and its divergent gene, their role in fatty acid resistance, and also characterize the effect of the SNP that lead to the identification of these two genes. First, I show that resistance to antimicrobial uFFAs is inducible in *S. aureus*. Second, *SAUSA300_2490* which I named *farR*, for a regulator of fatty acid resistance, is required for the inducible resistance to uFFAs. Third, *SAUSA300_2489* which I named *farE*, for an effector of fatty acid resistance, is a contributor to *S. aureus* persistence in the presence of antimicrobial uFFAs. Importantly, this *farE*-dependent

persistence is not only in the hyper-virulent MRSA, but also in methicillin-susceptible (MSSA) background. Furthermore, I show that FarE is an efflux pump in which its inactivation results in elevated uptake of $[^{12}C]$linoleic acid into bacterial cells. Fourth, I show that a SNP that resulted in $His_{121}Tyr$ substitution in the regulator FarR is alone sufficient to promote increased resistance to bactericidal levels of uFFAs. Unexpectedly, the growth analyses and reporter-gene assays of this chapter show that FarR is required for *farE* expression; a phenotype unusual for a typical TFRs. To follow up on this phenotype, I constructed an in-frame, markerless deletion of both *farR* and *farE* to conduct further studies on gene expression and structure-function relationships as I utilized transposon insertions mutants in all analyses of chapter 2.

In chapter 3, my aim was to characterize FarR, its regulatory function and its operator sites. First, using USA300Δ*farER* I show that *farE* cannot be induced in the absence of FarR, thus confirming that FarR is a TetR family activator. Second, I show that, unlike the staphylococcal QacR, FarR is auto-regulatory by binding to an operator site spanning its transcription start site. Third, I utilize electrophoretic mobility shift assays to examine the binding of His-tagged recombinant FarR to DNA operators with varying lengths and locations. I show that FarR utilizes three operator sites located in the *farER* intergenic segment to exert its regulatory functions. One operator spans the -10 core promoter element of P*farR* as well as its transcription start site, and represents the site of auto-repression of *farR*. Another operator that is located immediately downstream of the predicted transcription start site of *farE*, and represents a potential site of repression of both *farR* and *farE*. A third operator is located upstream of P*farE*, and represents the potential site of activation of *farE*. Interestingly, I also show that although the $His_{121}Tyr$ substitution in FarR is not physically located within its N-terminal DNA binding domain, it still affects DNA binding affinity and causes a relief to the auto-regulation. I also examine whether the FarR-dependent regulation of the fatty acid efflux pump FarE is linked to the mechanisms of fatty acid detoxification in *S. aureus*. It is established in the literature that fatty acid kinase deficiency in *S. aureus* promotes hyper-virulence in murine models of skin infection. I also show that this virulence is *farE*-dependent *in vitro* and *in vivo*, and that fatty acid kinase deficient mutants exhibit elevated constitutive

levels of *farE* that cannot be induced by exogenous fatty acids; an intriguing phenotype that contributes to what is established in the literature regarding *S. aureus* pathogenesis.

4.2   Limitations and future studies

FarR is a novel TFR that functions as a repressor in the absence of a fatty acid inducer, while acting as an activator in its presence. The transcription of *farE* increases in response to linoleic and arachidonic acids, and gene fusions demonstrated that expression of *farE* is induced only in the presence of functional FarR. Yet mobility shift assays show that FarR binds with similar affinities to OP_{Rep} and OP_{Act}. These findings raise two questions that my studies could not address; first, what is the mechanism by which FarR activates *farE*? And second, what is the ligand mediating this activation?

There are very limited reports of TFRs that exert positive-regulatory roles. DhaS, for example, is an unconventional TFR that functions as an activator (1). It binds an operator site upstream of the *dha* promoter, not to directly de-repress its target gene, but rather to bind a transcriptional co-activator, DhaQ. DhaQ then forms a complex with DhaS and subsequently activates transcription. In this situation, it is the transcriptional co-activator DhaQ which binds the physiologic ligand, dihyroxyacetone. Consequently, the TFR DhaS will only form a complex with the ligand-bound form of the transcriptional co-activator DhaQ (1). In consideration of this example, it is possible that FarR requires a co-activator that is yet to be determined; potentially either FakA and/or FakB2.

Perhaps identification of the endogenous ligand of FarR can aid in determining the exact mode of its regulation. TFRs typically bind hydrophobic ligands. Crystallography studies of FadR of *B. subtilis,* for example, show that acyl-CoAs are often co-crystallized with the protein from the *E. coli* host (2). Furthermore, FarR binding to OP_{Rep} is partially reduced in the presence of linoleoyl-CoA and arachidonoyl-CoA; however, it cannot be concluded that acyl-CoAs are the endogenous ligand for FarR. It is most likely that phosphorylated fatty acids are the ligand modulating the binding of FarR to its operator sites. A *fakA*-deficient mutant exhibit elevated constitutive levels of *farE* that cannot be further induced, due to the fact that fatty acids cannot be phosphorylated in the absence of this kinase. Purification of the fatty acid kinase proteins and the subsequent manual

phosphorylation of fatty acids for the use in mobility shift assays can help in confirming that phosphorylated fatty acids are the signal for regulation of *farE* via *farR*. Additionally, work is in progress to express FarR with a C-terminal 6×His tag in *S. aureus*. With this reagent in place, we could purify 6His-FarR from lysates of *S. aureus* grown in presence or absence of linoleic acid, and then conduct mass spectrometry to identify any protein or small molecule ligands that are captured in complex with FarR.

S. aureus can only synthesize branched- and straight-chain fatty acids as its genome does not code for any membrane phospholipid desaturase for synthesis of unsaturated fatty acids (3). Instead, the bacterium employs the fatty acid kinase (Fak) machinery to utilize host-derived uFFA for incorporation into the otherwise energetically expensive membrane lipid components (phospholipids, lipoproteins and cardiolipin) as well as lipoteichoic acids (4–6). A recent report by Lopez *et al.* has demonstrated that a Type VII secretion system (T7SS), which is crucial for prolonged bacterial survival and persistence in abscesses, is activated by uFFAs such as linoleic and arachidonic acids (7). Additionally, this activation is contingent on the incorporation of uFFAs into phospholipids and lipoprotein by the Fak machinery. This incorporation and subsequent stimulation of T7SS are required for virulence of *S. aureus* in a murine bacteremia model (7). Our data also provide evidence of a connection between the efflux pump FarE and fatty acid kinase FakA *in vitro* and *in vivo*. Since exogenous uFFA utilization by Fak machinery is central for *S. aureus* virulence, tight transcriptional regulation is then required to permit incorporation of uFFA into phospholipids, and induction of *farE* only when the metabolic capacity for incorporation into phospholipid is exceeded. Therefore, it is beneficial for the bacterium to fine tune the expression of *farE* such that it is induced rapidly when the cellular threshold for uFFA incorporation into phospholipid is exceeded, and subsequently represses the pump's expression once the fatty acid concentration falls below that threshold. This may explain the complex FarR-mediated regulatory mechanism of FarE, to ensure that the efflux pump is only expressed when needed. This also may explain the observation that FarR binds with similar affinities to OP_{Rep} and OP_{Act} as well as both FarR and the variant FarR7 bind equally well to OP_{Act}. Perhaps FarR binds constitutively to OP_{Act} but the binding to OP_{Rep} is what modulates the tight

regulation of *farE* expression. Therefore, in the absence of inducing ligand, binding to OP_{Rep} can repress *farE* and in the presence of inducing ligand, binding to OP_{Rep} is reduced, and binding to OP_{Act} can then contribute to activation of *farE* (Fig. 4.1). Further, analysis of the putative *farE* promoter reveals a GC-rich spacer between the predicted -10 and -35 promoter elements. This spacer, as opposed to a more typical AT-rich spacer in strong promoters, is indicative of reduced promoter activity that requires a transcriptional activator to help recruit RNA polymerase and the transcription machinery (8), further supporting the requirement for FarR as an activator of *farE*.

Reporter-gene assays show that FarR auto-represses its own expression, independent of the addition of fatty acid ligand. This raises the question as to why *farR* expression cannot be de-repressed in the presence of fatty acid inducer, while *farE* expression is de-repressed. Perhaps FarR does not directly require ligand binding, but rather employs a more complex regulatory mechanism that senses the fatty acid pool in the cell. The TFR AmtR, for instance, is involved in a post-transcriptional regulation of urea metabolism without binding to any effector molecule (9). This regulation utilizes a sensory system that involves a trans-acting sRNA in response to nitrogen availability. When nitrogen is overabundant, the sRNA targets *amtR* mRNA and blocks its translation. Under nitrogen starvation the sRNA is down-regulated and *amtR* is successfully translated. The AmtR repressor then works in combination with the global nitrogen activator, GlnR, to fine-tune the expression of urea-degrading metabolic pathway (9). Additionally, LuxR of *V. harveyi* that deviates from the norm of TFRs and functions as both repressor and activator depending on the presence or absence of a modulating ligand, is also subject to sRNA-mediated regulation to ensure tight post-transcriptional control of quorum sensing genes (10). Small RNAs can be encoded on the opposite strand of target mRNA, and despite the complementarity, they block translation. These antisense RNA molecules often target 5′ untranslated regions (UTR) that are close to or overlapping the ribosomal binding site of target genes (11–13). Therefore, it is possible that FarR employs tight post-transcriptional regulatory mechanism to ensure that the efflux pump is only expressed when needed. Not only the spatial arrangement of P_{farR} and P_{farE} on the plus and the minus strands conform to the typical antisense and target mRNA paradigm, but also analysis of putative P_{farE} did

reveal a 5′-UTR, upstream of the ribosomal binding site of *farE,* that is indicative of post-transcriptional regulation (Fig. 4.2). Further, analysis of P*farR* revealed an even longer 5′-UTR, compared to that of *farE,* that also suggests a possible post-transcriptional regulation of *farR* (Fig. 4.2). Structural and functional studies will be needed to further shed light on the exact regulatory mechanism of FarR and its mechanism of de-repression and *farE* activation.

In conclusion, this thesis reports the first description of a specific mechanism of inducible resistance to antimicrobial fatty acids in a Gram-positive pathogen, and the first staphylococcal TFR that functions as an activator and a repressor. It is evident that one of the strategies *S. aureus* employs to cope with antimicrobial fatty acids and persist successfully on a human host is via a complex regulatory mechanism where a TFR with dual functionality controls the expression of a fatty acid specific- efflux pump.
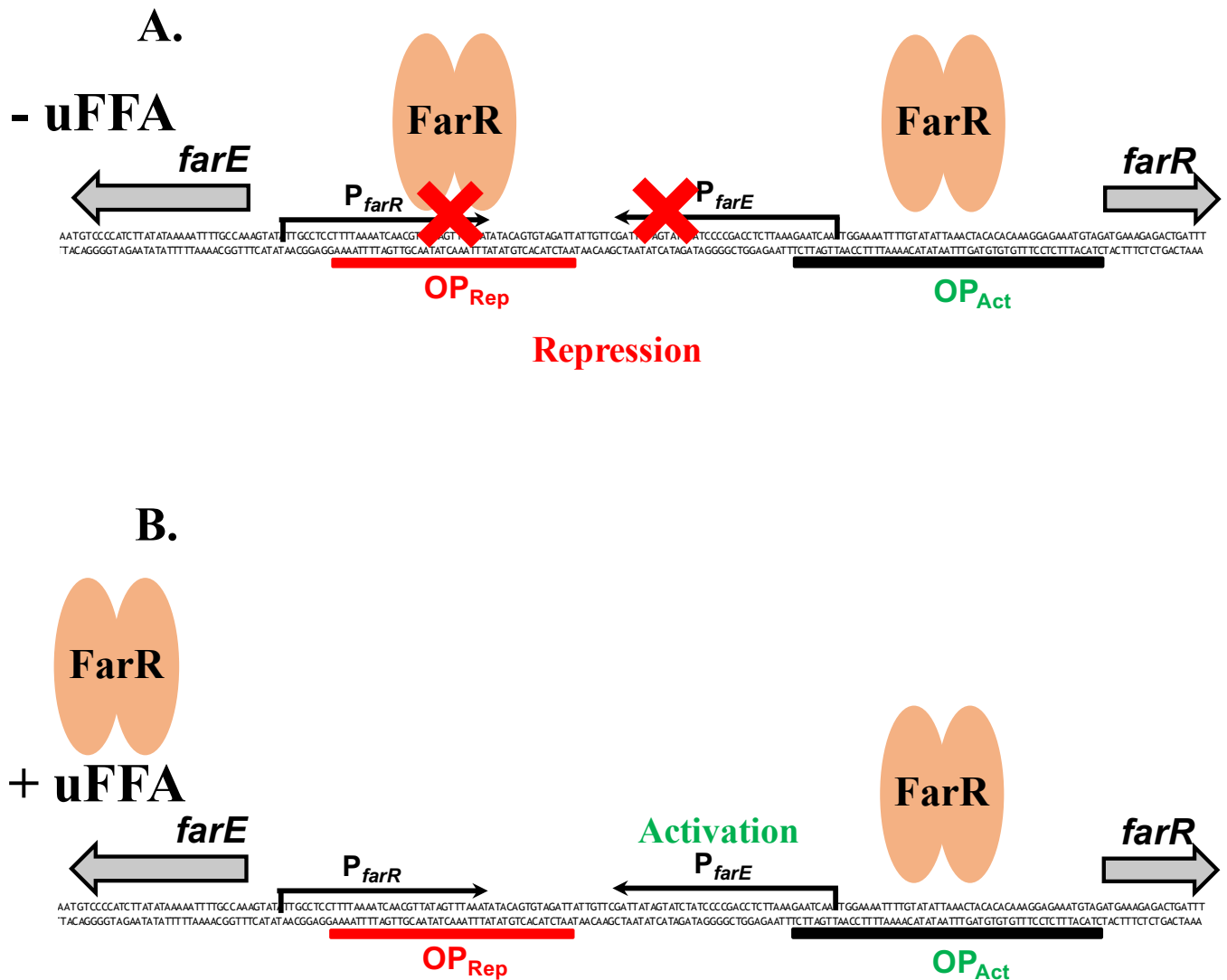
Figure 4.1 Schematic model of the potential mechanism of P*farE* regulation by FarR, depending on exogenous fatty acid ligand. FarR binds constitutively to OP_Act but the binding to OP_Rep is what modulates the regulation of *farE* expression. **A**. In the absence of inducing ligand, FarR binds to OP_Rep, thereby blocking transcription from P*farE*. **B**. In the presence of inducing ligand, binding to OP_Rep is reduced, and binding to OP_Act can then lead to activation of *farE*.
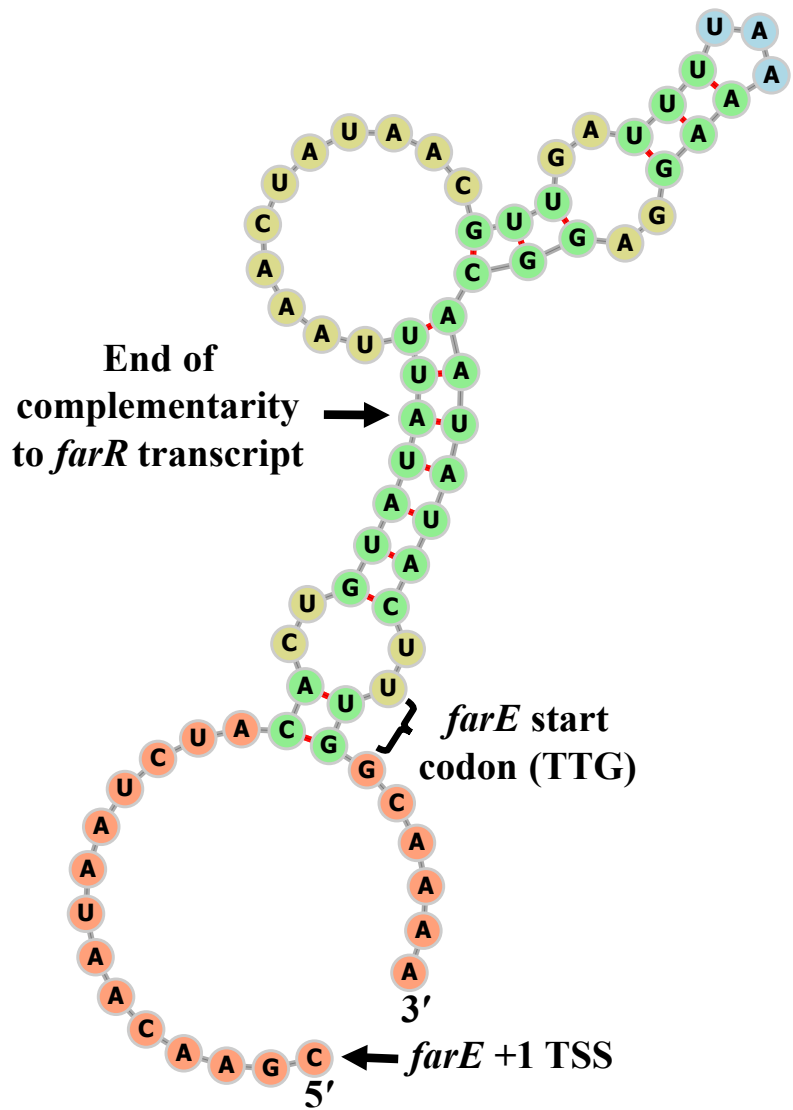
End of complementarity to *farR* transcript →

farE start codon (TTG)

farE +1 TSS ←

5′

3′

Green: Stems (canonical helices)
Red: Multiloops (junctions)
Yellow: Interior Loops
Blue: Hairpin loops
Orange: 5′ and 3′ unpaired region

Green: Stems (canonical helices)
Red: Multiloops (junctions)
Yellow: Interior Loops
Blue: Hairpin loops
Orange: 5′ and 3′ unpaired region

farR start codon (ATG)

farE +1 TSS (Complement)

farR +1 TSS

5′

3′
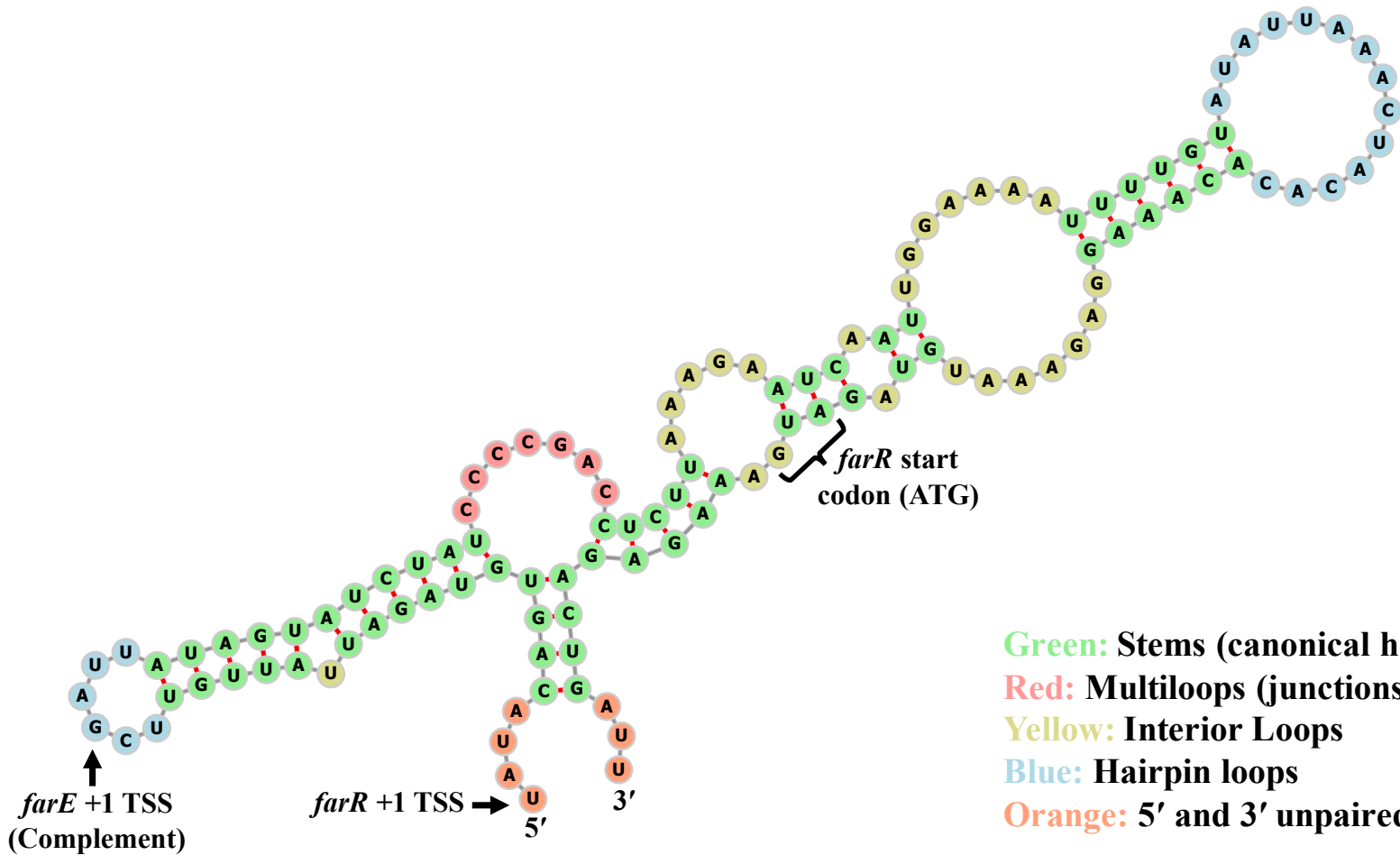
Figure 4.2 Secondary structure prediction of *farE* and *farR* RNA using RNAfold Webserver. The nucleotides are colored according to the type of structure that they are in. **A.** *farE.* **B.** *farR.*

4.3 References

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Appendices

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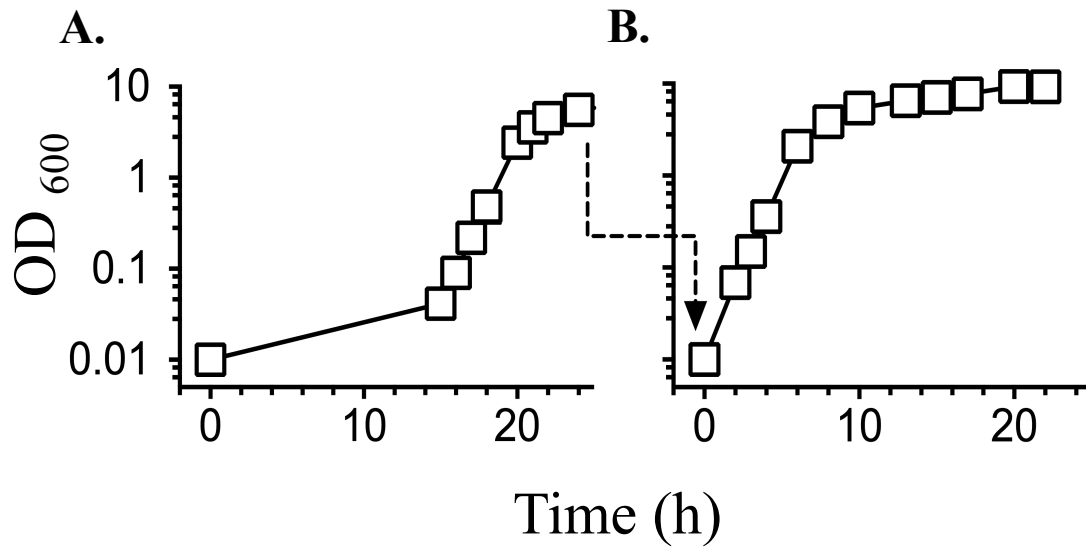
Appendix 2. Adaptation of *S. aureus* USA300 during growth in TSB + 50 µM linoleic acid. A. Cells from overnight culture grown in TSB were inoculated into TSB + 50 µM linoleic acid, to achieve OD_{600} = 0.01. **B.** Cells from stationary phase of the primary culture were inoculated into fresh TSB + 50 µM linoleic acid
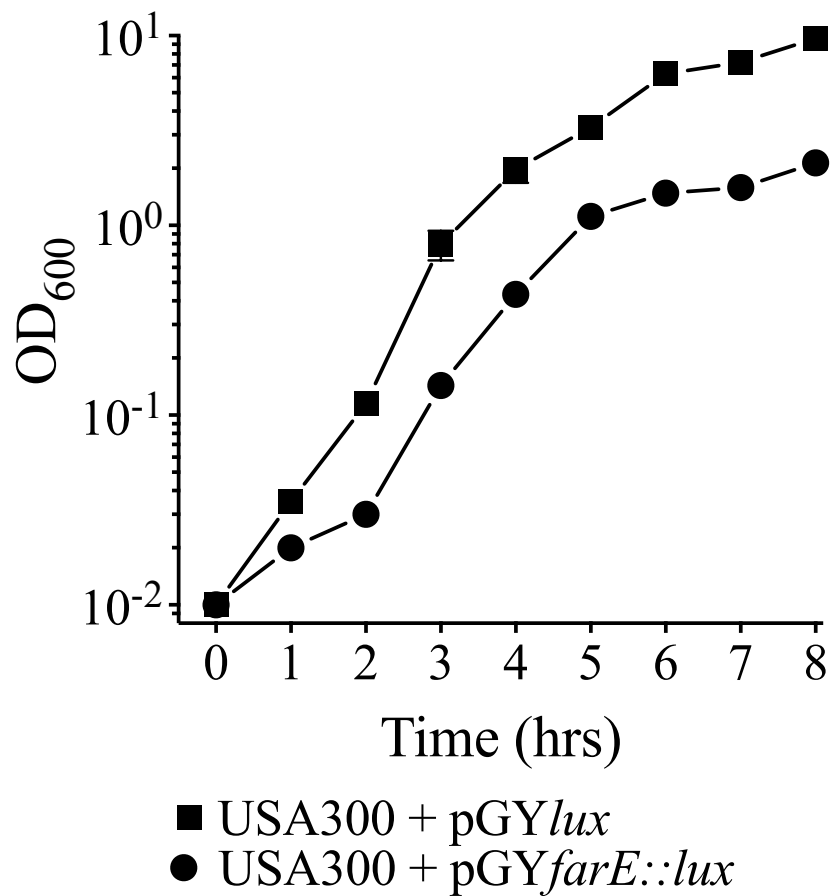
Appendix 3. Differential effect of pGYlux and pGYfarE::lux on growth of *S. aureus* USA300 in TSB + 20 μM linoleic acid. Triplicate flasks of USA300 harboring pGY*farE:lux*, or empty pGY*lux* vector were inoculated into TSB + 20 μM linoleic acid, and growth (OD_{600}) was monitored on an hourly basis.
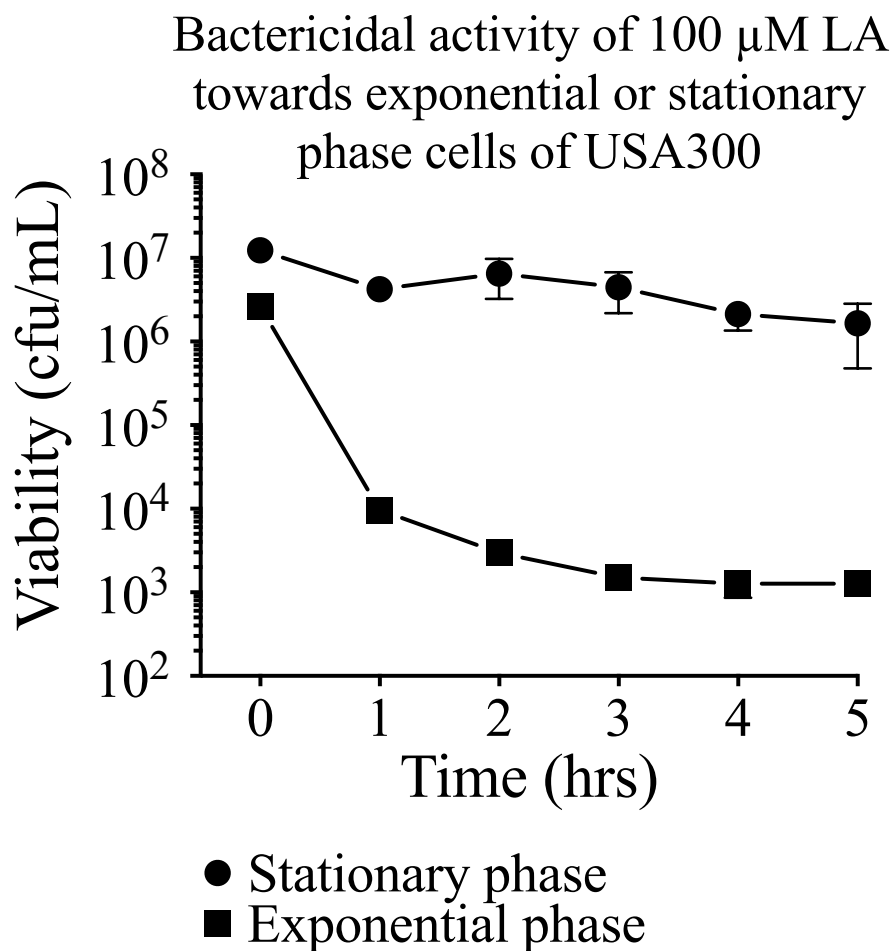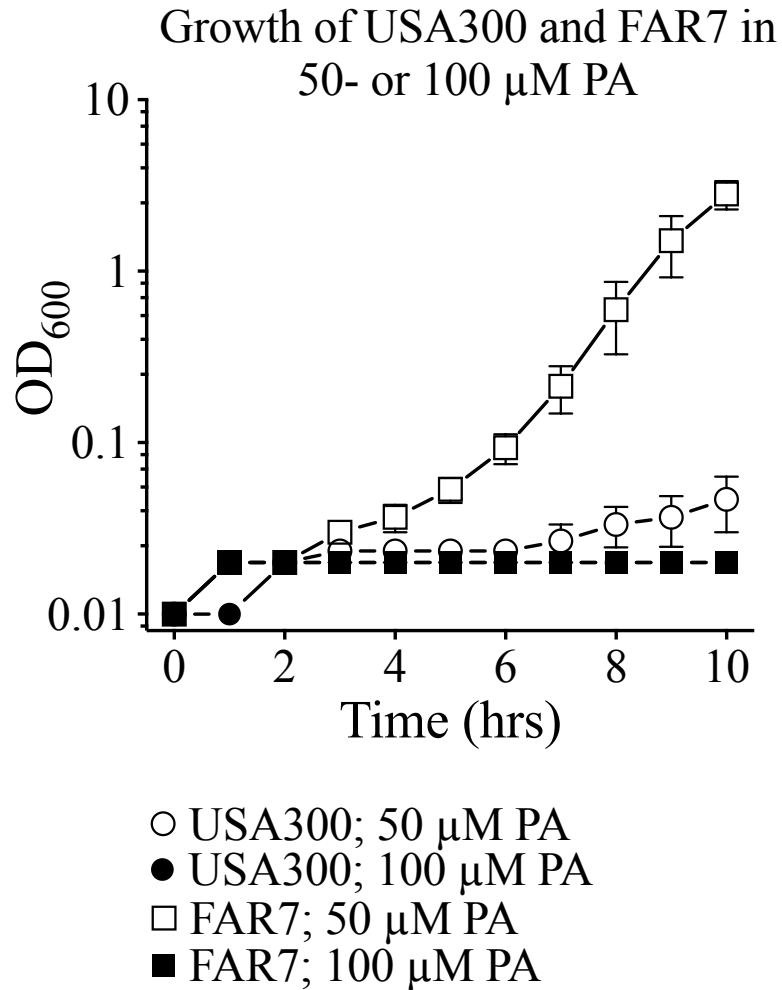
Appendix 4. Exponential phase cells are highly susceptible to the bactericidal activity of 100 μM linoleic acid (LA). USA300 cells from an overnight culture (stationary phase) or mid-exponential phase culture (OD_{600} = 0.5) were inoculated to achieve OD_{600} = 0.01, in TSB containing 100 μM LA, and viability was monitored at hourly intervals. Each data point represents the mean and standard deviation of triplicate cultures.
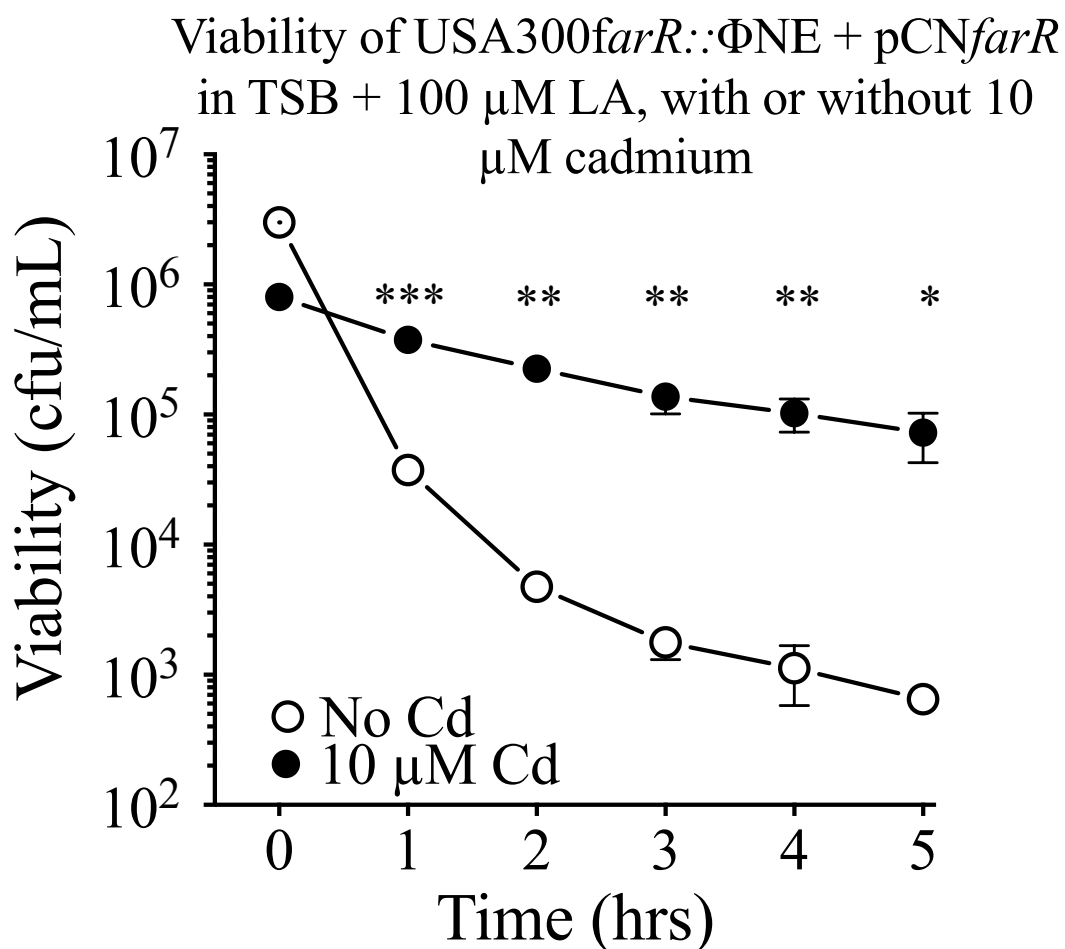
Appendix 5. Cadmium inducible expression of farR protects USA300*farR*::ΦNE from the bactericidal activity of 100 μM linoleic acid. For challenge without Cd, USA300*farR*::ΦNE + pCN*farR* was grown to mid-exponential phase in TSB + 20 μM linoleic acid, and then sub-cultured to OD_{600} = 0.01 in TSB + 100 μM linoleic acid, followed by monitoring of viability at hourly intervals. To induce *farR* expression, USA300*farR*::ΦNE + pCN*farR* was grown to mid-exponential phase in TSB + 20 μM linoleic acid and 10 μM Cd, followed by subculture into TSB + 100 μM linoleic acid and 10 μM Cd. Each data point represents the mean and standard deviation of quadruplicate flasks. Significant differences in viability at each time point were determined by unpaired one-tailed Student's t-test; ***, $P < .001$; **, $P < 0.01$; *, $P < 0.05$

Growth of USA300 and FAR7 in 50- or 100 μM PA

○ USA300; 50 μM PA
● USA300; 100 μM PA
□ FAR7; 50 μM PA
■ FAR7; 100 μM PA

Appendix 6. FAR7, but not USA300, is able to grow in TSB containing 50 μM palmitoleic acid (PA). Stationary phase cells of USA300 or FAR7 were inoculated into TSB containing 50 or 100 μM palmitoleic acid, and growth (OD_{600}) was monitored at hourly intervals. Each data point represents the mean and standard deviation of triplicate cultures.
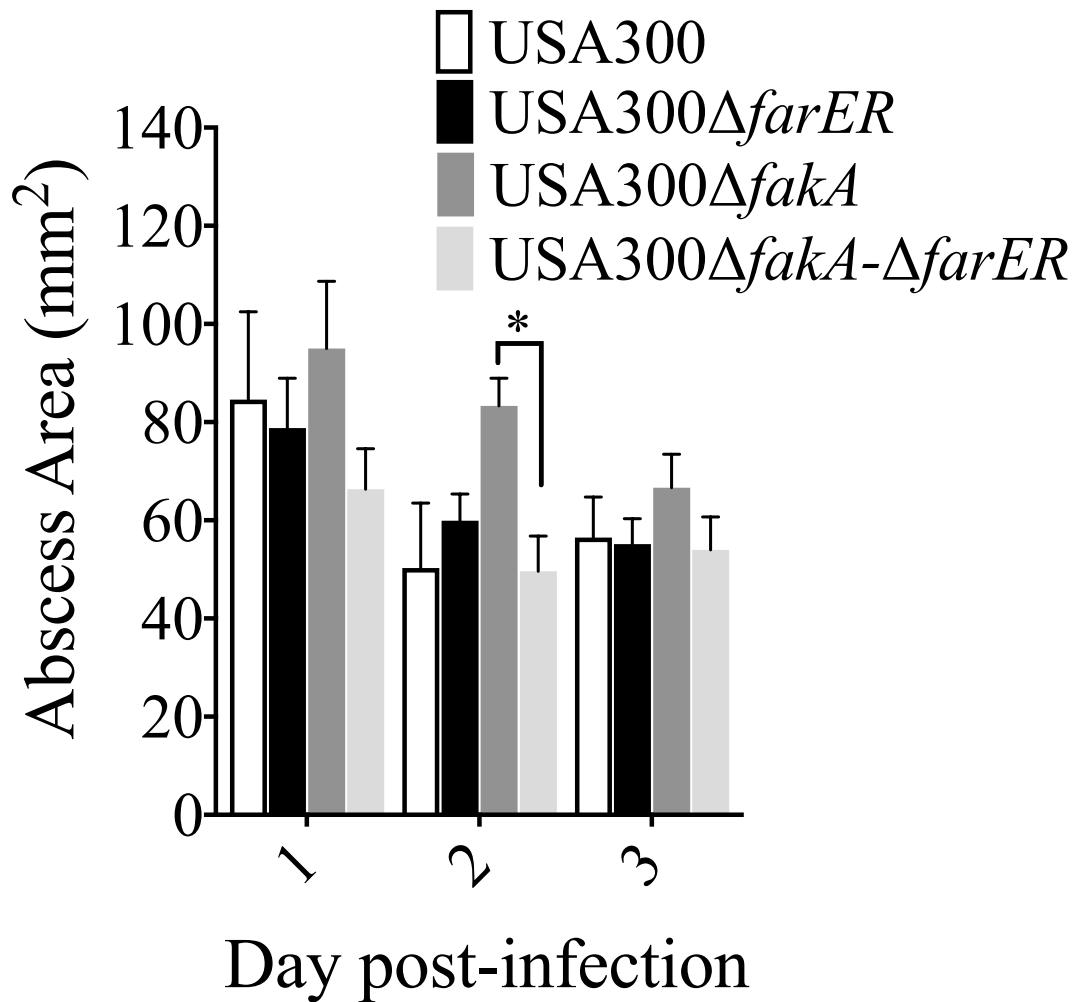
Appendix 7. ***fakA-farER* influence virulence in a murine skin abscess model of infection**. 6-week female BALB/c mice were infected with $2x10^7$ CFU of USA300, USA300*ΔfarER*, USA300*ΔfakA* and USA300*ΔfakA-farER*. Assesses were imaged daily and surface area was quantified using ImageJ. The reduction in virulence exhibited by USA300*ΔfakA-farER* is significant relative to that of USA300*ΔfakA* as determined by a two-way ANOVA where $*P < 0.05$. Error bars represent Standard Error of the Mean. n=8 abscesses per strain.

A.

B.
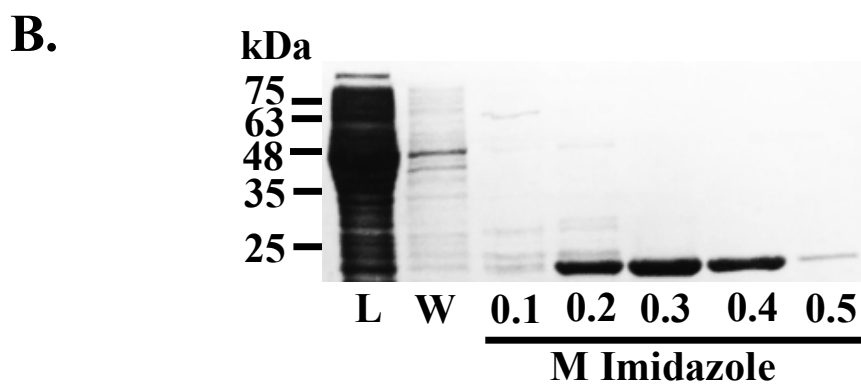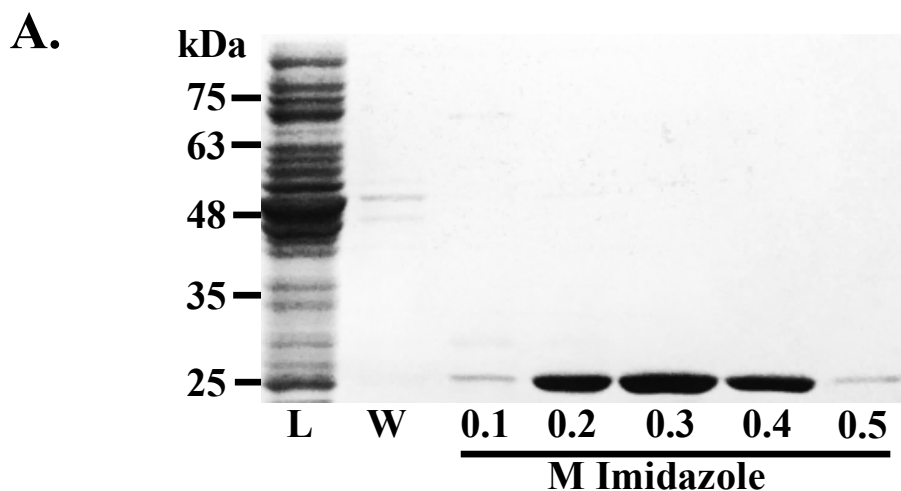
Appendix 8. Purification of 6×His-tagged FarR from wildtype USA300 and the variant FAR7 using nickel affinity chromatography. Cell lysate was applied onto a 1 mL His-Trap nickel affinity column that was equilibrated with binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 40 mM imidazole, pH 7.4). After washing with binding buffer, bound His-tagged protein was eluted over a linear imidazole gradient (0.1-0.5 M) in 20 mM sodium phosphate. Column fractions were assessed by SDS-polyacrylamide gel electrophoresis to check for purity. L is the unbound fraction from the column, and W is the washed flow through fraction. Purified FarR protein band is at ~ 23 kDa. **A.** Native FarR and **B.** Variant FarR7.

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Publications

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Manuscripts in Preparation

Alnaseri, H., Ferguson, K.A., Schneider, J.E.T., Heinrichs, D.E., and McGavin, M.J. 2017. Identification of FarR binding site responsible for auto-repression and efflux-mediated resistance to antimicrobial fatty acids in *Staphylococcus aureus*.

Invited Podium Presentations

Alnaseri, H., and McGavin, M.J. Identification of FarR binding site responsible for auto-repression and efflux-mediated resistance to antimicrobial fatty acids. Oral presentation at the International Conference on Gram Positive Pathogens, Nebraska Omaha USA, October 11, 2016

Alnaseri, H., and McGavin, M.J. Identification of *farR* and *farE* as a regulator and effector of *Staphylococcus aureus* resistance to antimicrobial fatty acids. Oral platform presentation at London Health Research Day, London Ontario, March 29, 2016

Alnaseri, H. Identification of *farR* and *farE* as a regulator and effector of *Staphylococcus aureus* resistance to antimicrobial fatty acids. Oral presentation at the student symposium competition of the Canadian Society of Microbiologists, Regina Saskatchewan, June 16, 2015

Poster Presentations

Alnaseri, H., and McGavin, M.J. Identification of FarR binding site responsible for auto-repression and efflux-mediated resistance to antimicrobial fatty acids. Poster presentation at the Canadian Society of Microbiologists meeting, Waterloo Ontario, June 21, 2017

Alnaseri, H., and McGavin, M.J. Identification of FarR binding site responsible for auto-repression and efflux-mediated resistance to antimicrobial fatty acids. Poster presentation at London Health Research Day, London Ontario, March 28, 2017

Alnaseri, H., Arsic, B., Schneider, J.E., Kaiser, J.C., Heinrichs, D.E., and McGavin, M.J. Identification of *farR* and *farE* as a regulator and effector of *Staphylococcus aureus* resistance to antimicrobial fatty acids. Poster presentation at the Infection and Immunity Research Forum, London Ontario, November 6, 2015

Alnaseri, H., Arsic, B., Schneider, J.E., Kaiser, J.C., Heinrichs, D.E., and McGavin, M.J. Identification of *farR* and *farE* as a regulator and effector of *Staphylococcus aureus* resistance to antimicrobial fatty acids. Poster presentation at the Combined Infectious Diseases and Microbiology/Immunology Educational Retreat, Grand Bend Ontario, May 21,2015

Alnaseri, H., Arsic, B., Schneider, J.E., Kaiser, J.C., Heinrichs, D.E., and McGavin, M.J. Identification of *farR* and *farE* as a regulator and effector of *Staphylococcus aureus* resistance to antimicrobial fatty acids. Poster presentation at London Health Research Day, London Ontario, April 1, 2015

Alnaseri, H., Arsic, B., Schneider, J.E., Heinrichs, D.E., and McGavin, M.J. Identification of *farR* and *farE* as a regulator and effector of *Staphylococcus aureus* resistance to antimicrobial fatty acids. Poster presentation at the Infection and Immunity Research Forum, London Ontario, November 6, 2014

Alnaseri, H., Arsic, B., Schneider, J.E., Heinrichs, D.E., and McGavin, M.J. Identification of *farR* and *farE* as a regulator and effector of *Staphylococcus aureus* resistance to antimicrobial fatty acids. Poster presentation at the International Symposium on Staphylococci and Staphylococcal Infection, Chicago Illinois USA, August 26-29, 2014

Alnaseri, H., Lee, J., and McGavin, M.J. The role of *tetR* transcriptional regulator in the response of *Staphylococcus aureus* to antimicrobial unsaturated fatty acids. Poster presentation at London Health Research Day, London Ontario, March 18, 2014

Alnaseri, H., Lee, J., and McGavin, M.J. The role of *tetR* transcriptional regulator in the response of *Staphylococcus aureus* to antimicrobial unsaturated fatty acids. Poster presentation at the Infection and Immunity Research Forum, London Ontario, November 1, 2013

Alnaseri, H., Sheldon, J., Heinrichs, D.E., and McGavin, M.J. The influence of unsaturated free fatty acids on the expression of cell surface proteins in Community-Associated Methicillin Resistant *Staphylococcus aureus* (CA-MRSA). Poster presentation London Health Research Day, London Ontario, March 19, 2013

Alnaseri, H., Sheldon, J., Heinrichs, D.E., and McGavin, M.J. The influence of

unsaturated free fatty acids on the expression of cell surface proteins in Community-Associated Methicillin Resistant *Staphylococcus aureus* (CA-MRSA). Poster presentation at the Infection and Immunity Research Forum, London Ontario, November 30, 2012

Community Involvement

2017	**National Scholarship Ranking Participant**
	Faculty of Science, Western University
2016	**Poster Competition Judge**
	Infection and Immunity Research Forum
2016- 2017	**Microbiology and Immunology Graduate Students Representative**
	The Canadian Society of Microbiologists
2015-2017	**Microbiology and Immunology Graduate Students Representative**
	Schulich School of Medicine and Dentistry Graduate Student Council
2012-2017	**Member of the Organizing Committee**
	Infection and Immunity Research Forum
2012-2016	**Community Volunteer**
	Let's Talk Science
2012-2014	**Children's Hospital Subcommittee Member**
	Strong Bones, Strong Muscles, Strong Minds
2009	**Student Leader**
	Thames Valley District School Board